Mitigating Selenium Ecotoxic Risk by Establishment of a Model Aquatic Ecosystem

2011 Final Report

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INTRODUCTION

Red Rock Ranch (RRR) is a 600+ acre farm located at Five Points, CA that practices Integrated On-Farm Drainage Management (IFDM). This integrated system cycles drainage water through a series of salt tolerant crops with the objective of reusing the saline water to produce marketable crops while managing the high salinity drainage water directly on site. IFDM is a promising strategy for reducing the volume of contaminated water that is often produced as a consequence of irrigation in the San Joaquin Valley. Unfortunately, soils in the San Joaquin Valley are rich in Se, which is leached into the drainage water in the IFDM process. Further concentration of the drainage water by evaporation results in final water contaminated with toxic levels of Se.

Microphytes isolated from evaporation basins in the Tulare Lake Drainage District (TLDD) have been shown to be active in Se volatilization, resulting in sizeable depletions of water-column Se concentrations. Rates of Se volatilization and the extent of bioaccumulation have been shown to vary between microphyte species. Fertilizer inputs and selective macroinvertebrate grazing, specifically by the brine shrimp (*Artemia franciscana*), are also known to affect microphyte composition. In this project, our primary objective was to optimize the conditions for maximal Se removal by Se volatilization and brine shrimp harvest. In 2010, we continued to test the intensive *Artemia* farming system consisting of a paddlewheel driven racetrack pond. We expanded the research scope at RRR for testing variable conditions with the introduction of eight 100 L mesocosms. Due to lack of high-salinity sump water, and water loss from the racetrack pond due to seepage, the focus shifted from the primary pond to the mesocosms in the summer of 2010.

OBJECTIVES

The overall objective of this project is to establish a model aquatic ecosystem that optimizes total Se remediation and subsequently reduces ecotoxic risk though the combination of natural Se volatilization and food chain disruption. The stated aims of this project were as follows:

- Monitor microphyte community composition and correlate changes in *chlorophyll a* concentrations, Se volatilization and brine shrimp population status with physical parameters such as salinity and water chemistry
- Assess effects of water chemistry alteration on algal dynamics for optimizing the production of brine shrimp, as well as Se volatilization
- Evaluate the ecotoxic status of the engineered algae-brine shrimp ecosystem
- Determine potential alternate uses of drainage water such as feed supplement production and biofuel feedstock production

Field Site

Background

The Red Rock Ranch selenium bioremediation test facility consists of a 90,000 L racetrack pond enclosed within plastic netting. The RRR racetrack pond was filled with a mixture of available concentrated drainage and aqueduct water to achieve a final salt concentration of approximately 30 ppt. At this salt concentration, the Se level was between 3.5-4 ppm. Drainage water was amended with nutrient slurry derived from a mix of chicken manure and straw. Phosphorus in the form of $Na_2H_2PO_4$ and iron as Fe-EDTA was added, in accordance with previous experience with RRR drainage water. Nutrients were added approximately every 3 months. Air and water temperature, water level, total dissolved solids (TDS), water density, electrical conductivity (EC), pH, oxidation-reduction potential (ORP), and dissolved oxygen (DO) were monitored several times per week. Samples for water Se concentration, microalgal tissue Se concentration and chlorophyll *a* concentration determination, as well as microscopy, microalgal DNA profiling by Denaturing gradient gel electrophoresis (DGGE), and volatile Se determination were carried out once a month. To allow testing simultaneous testing of variable conditions eight tubs of 100 L capacity each were installed at the facility and different nutrient additions were tested in these "mesocosms".

Mesocosms

To test the effect of various carbon and/or nitrogen source additions, eight mesocosms were installed in the racetrack pond. The mesocosms were conducted in the pond itself to provide environmental conditions as similar as those experienced by organisms in the larger pond, in particular to avoid excessive temperature fluctuations. As water for racetrack pond became unavailable due to drought conditions in June 2010, the focus of research at RRR shifted from the primary pond to the mesocosms. In order to continue research under production conditions, the mesocosms were relocated to a greenhouse at UC Davis at the beginning of December 2010.

Water Quality

Due to a combination of drought conditions and limited irrigation in the adjacent fields, we were not able to supplement the pond with highly concentrated IFDM drainage water to date. Water salinity in the pond ranged from 30-60 g/L (Figure A3). The higher salinities were observed only at very low water levels (300 mm). The very low salinity again contributed to an undesirable green algae (*Cladophora*) bloom. This filamentous algae is not suitable for brine shrimp feed. The filaments are too large for the shrimp to be able to consume them. Previous laboratory analysis has also indicated it does not show promise as potential biodiesel feedstock due to relatively low lipid content of *Cladophora*. Laboratory microcosm studies carried out in the first quarter of 2010 determined that upper threshold of *Cladophora* salinity tolerance is between 60 and 80 ppt (Table 1).

In order to manage the filamentous green algae bloom, we explored different options of biological control. We hoped to use tilapia fish (*Oreochromis mossambicus*) as a biological control mechanism for the algal bloom. These fish can survive in low quality water on diet of algae, and are relatively salt tolerant. Unfortunately these fish are

considered a pest species in parts of California, and we were not able to obtain a permit from the Department of Fish and Game to import them into the Central Valley.

Table 1. Filamentous algae response to increasing salinity. Filter sterilized RRR racetrack pond water amended with Na_2SO_4; experiment performed in triplicate.

Salinity (ppt)	Filamentous algae bloom
40	+
60	+
80	-
100	-

I. RRR Racetrack Pond Shrimp Production

Artemia franciscana numbers averaged >35 shrimp/L during June and July then fluctuated around a mean of approximately 20 shrimp/L during August, September, October (see Figure 1).

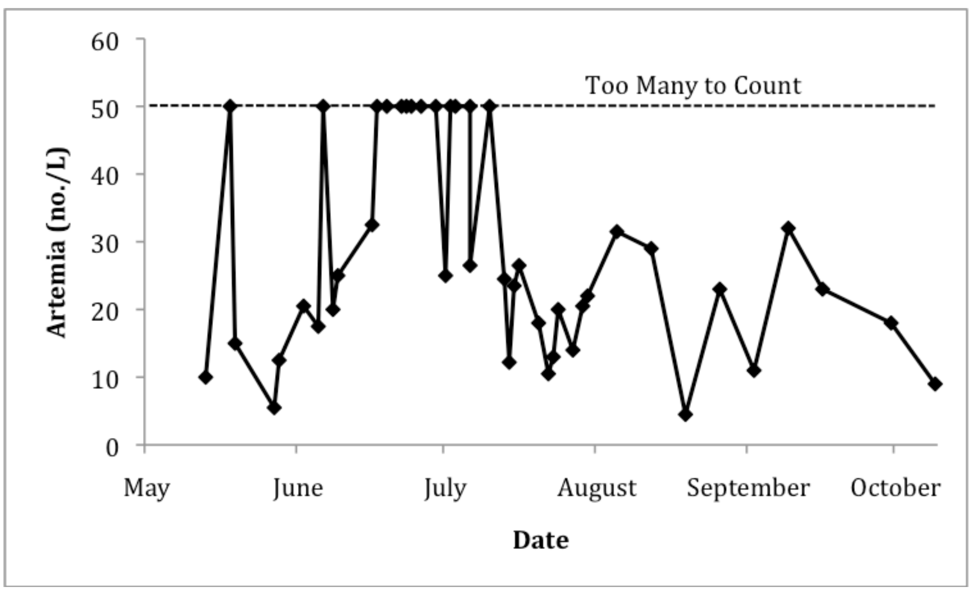

Figure 1. *A. franciscana* abundance in the pond (reported as number of adult shrimp/L). Due to difficulties in accurate counts at high densities, shrimp numbers over 50 shrimp/L were reported as “too many to count”.

The severity of the water situation at RRR resulted in low salt water additions to the pond taking place in three major events during the monitoring period (Figure A1). This resulted in large fluctuations in TDS, EC and salinity (Figure A3). While undesirable in a production environment, these fluctuations allowed us to determine the effect of large scale salinity changes on the *A. franciscana* population. The rapid drops in TDS due to water dilution with each addition are clearly linked to significant decreases in *A. franciscana* numbers (Figure 2). While water temperature remained relatively constant for most of the monitoring period, the drop in temperature below 20^{o}C in October is likely correlated with the drop in shrimp numbers in this period (Figure 3). The third physical characteristic that shows correlation with shrimp numbers is pH. While total pH and total shrimp numbers do not demonstrate any obvious relationship, changes in pH appear to elicit congruent shifts in shrimp population numbers, with increases in pH preceding by a few days increases in shrimp numbers and decreases in pH followed by decreases in shrimp numbers (Figure 4). Unlike previous years no correlation between temperature and shrimp numbers was found.

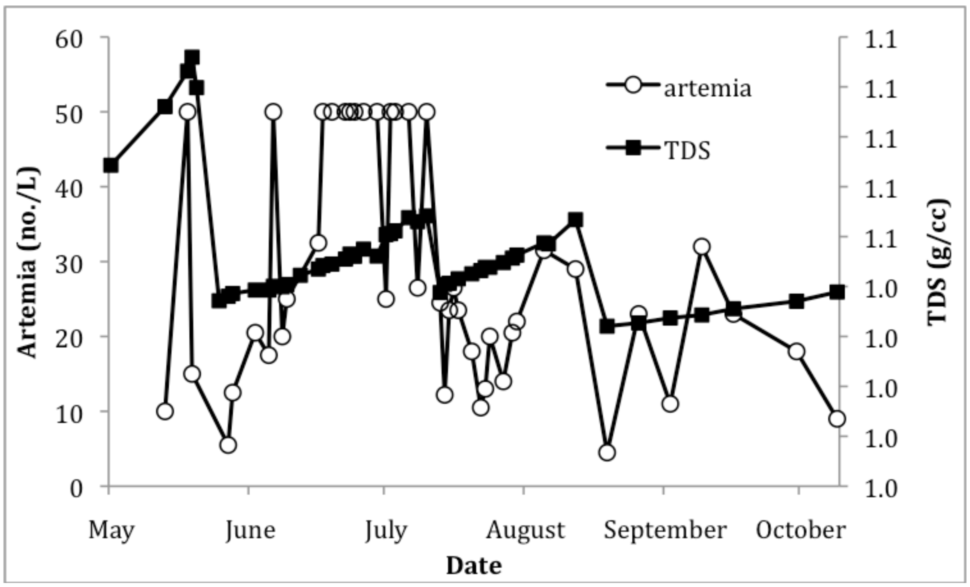

Figure 2. Effect of TDS on *A. franciscana* abundance. Shrimp number decreased sharply following each drop in TDS (i.e. fresh water addition).

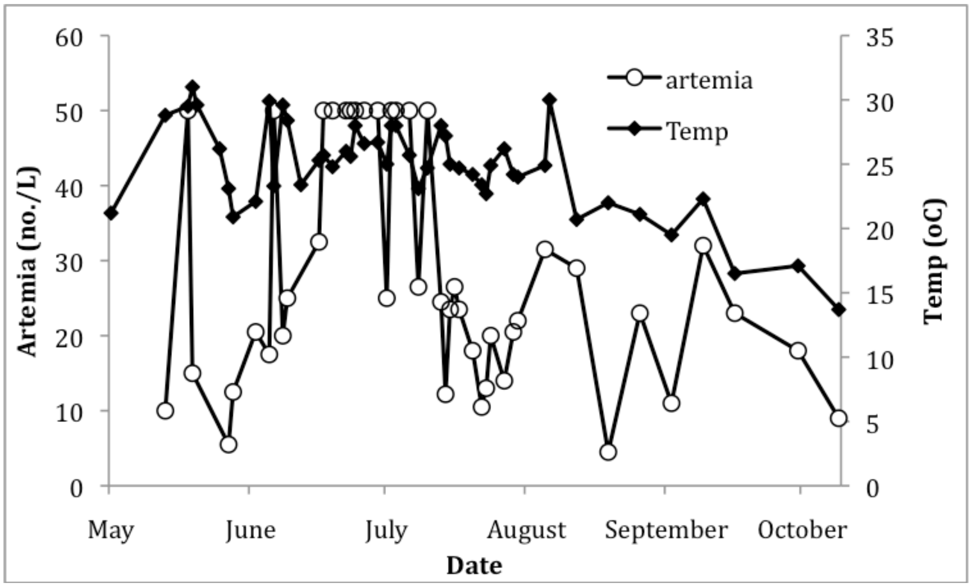

Figure 3. Effect of water temperature on *A. franciscana* abundance. Shrimp number decrease appears to be correlated with temperature below 20^{o}C.

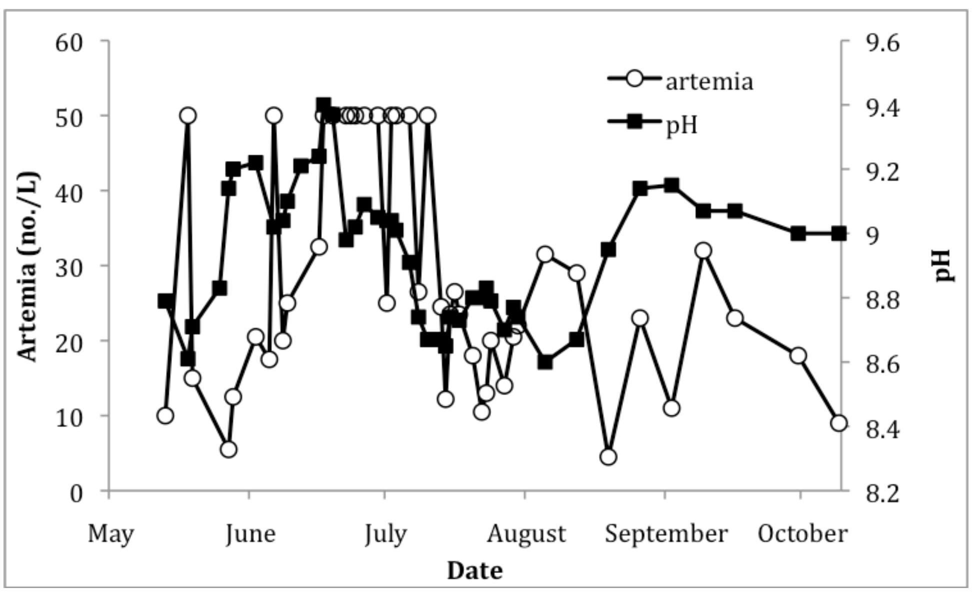

Figure 4. Effect of pH on *A. franciscana* abundance. Shrimp number changes appear to follow pH changes.

II. Mesocosm Experiment

Objectives

The aim of this experiment was to examine effects of additional carbon and nitrogen in different forms on algal growth and diversity and brine shrimp production.

Background

Eight mesocosms were set up at the RRR facility. 100 L barrels were used to separate each mesocosm from the pond while maintaining the same environment factors, such as temperature and light exposure as in the pond. The main difference from pond environment was the mixing system in mesocosms. The mesocosms used air bubbling as mixing instead of paddle wheel in the pond. Since water to start the mesocosms was obtained directly from the pond, each mesocosm initially contained the same algal community and brine shrimp population as the pond.

On day one seven different nutrients regimes were started in the mesocosms. Glycerol was used representing organic carbon sources, whereas CO_2 gas representing inorganic form. The additional amount was based on reviewed information on algal culture. Glycerol was added to make the concentration in mesocosm to 0.1 g-C/L. CO_2 was applied until the pH reached 7.5. Nitrogen was added in 3 different forms, ammonium sulfate, urea, and casein. Amount of added nitrogen was calculated to 50 mg N/L except in casein treatment. There was no information available for casein N content, the estimation was made from a previous lab experiment - adding 1 g of casein per 1 L of water.

Sampling Parameters

Mesocosm water samples were collected before addition of nutrients and used as background water chemistry. Following the initial samples, water physical and chemical parameters were collected daily, weekly and/or monthly. Physical factors, i.e. pH, salinity, and temperature were measured daily. Chemical factors; TOC, DOC, Total N, N-NH_4^+, N-NO_3^-, P, and Fe were measured weekly. Water Se and Tissue Se were evaluated monthly. Biological effects on algal community were evaluated by measurement of changes in biomass based on chlorophyll A. Algal and microbial diversity were identified by PCR and Denaturing Gradient Gel Electrophoresis (DGGE). Brine shrimp productivity assessment was based on density of brine shrimp in numbers per L measured weekly.

Mesocosm Results

The results show no obvious correlation between algal biomass and the addition of organic carbon substrates, while CO_2 addition had a positive effect (Figure 5). The increasing of algal biomass after carbon dioxide addition was due to the increasing of dissolved inorganic carbon in the system (Figure 6). However, other factors such as grazing by shrimp might limit our observation of the actual increase of algal biomass in the mesocosm. The higher the number of shrimp, the lower the algal biomass detected (Figure 7).

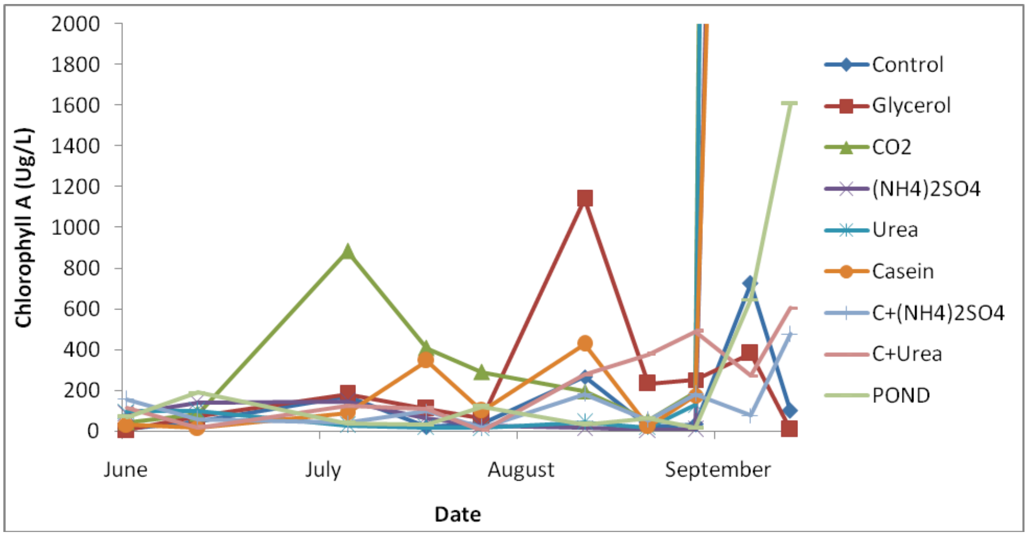

Figure 5. Algal biomass in different treatments (reported as chlorophyll A amount in $\mu g/L$). The data which are out of the range on the graph were resulted from the algal bloom in the tubs.

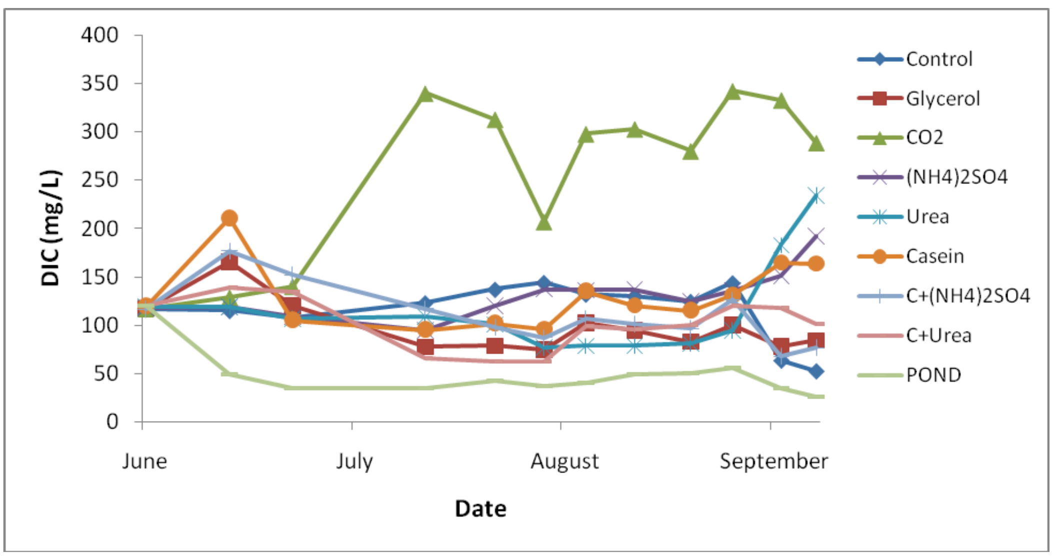

Figure 6. Dissolved inorganic carbon (DIC) in different treatments. The carbon dioxide addition significantly increased DIC in the mesocosm.

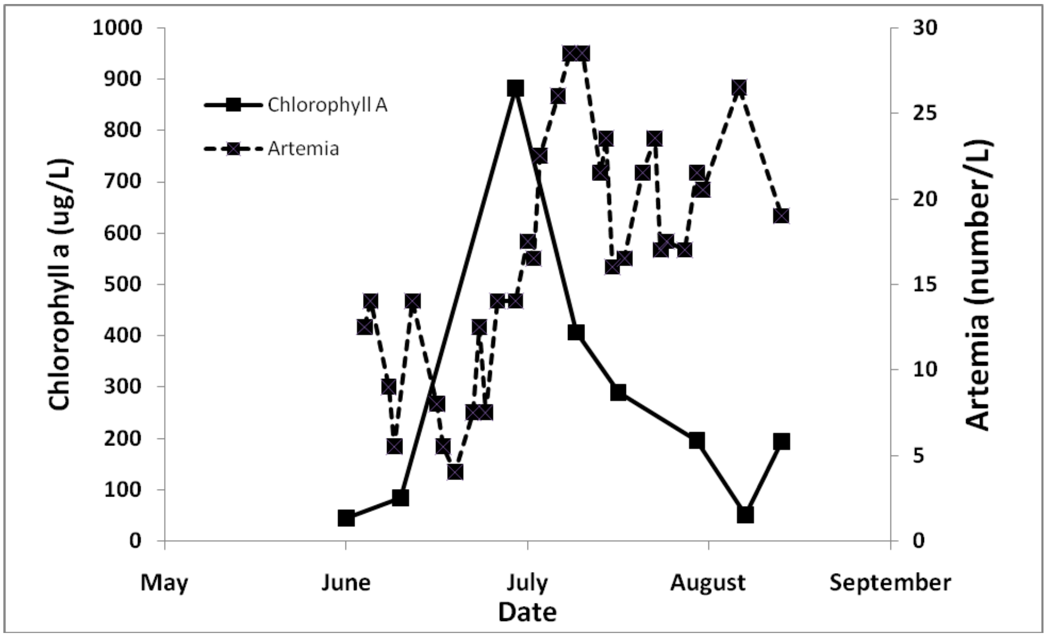

Figure 7. Relationship between algal biomass (as chlorophyll a) and A*rtemia. franciscana* abundance in carbon dioxide addition treatment. Following the increase of algal biomass, shrimp number also increased. Grazing by shrimp limited further algal biomass production.

The addition of nitrogen did not show a direct relationship to the change of algal biomass production or brine shrimp number; however, the different forms of nitrogen and carbon additions affected water chemistry. Nitrite level increased while nitrate level decreased in the treatments with organic carbon additions (Figure 4). The addition of carbon dioxide resulted in lower pH, thus increasing the solubility of iron and phosphorus in the water (Figure 9 and 10).

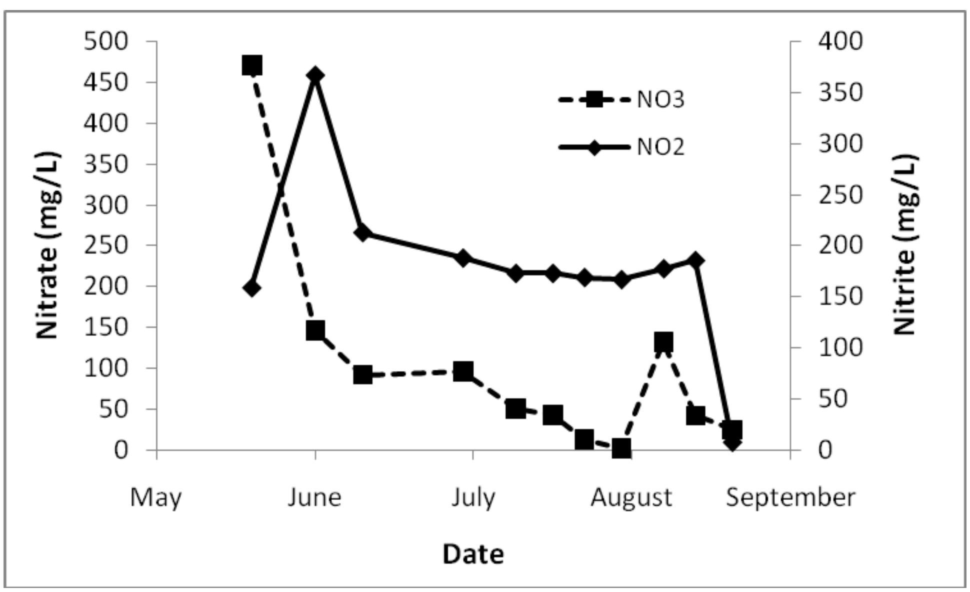

Figure 8. Nitrite and nitrate concentration in organic carbon (glycerol added) treatment. The nitrite level increased while nitrate level decreased after addition of organic carbon.

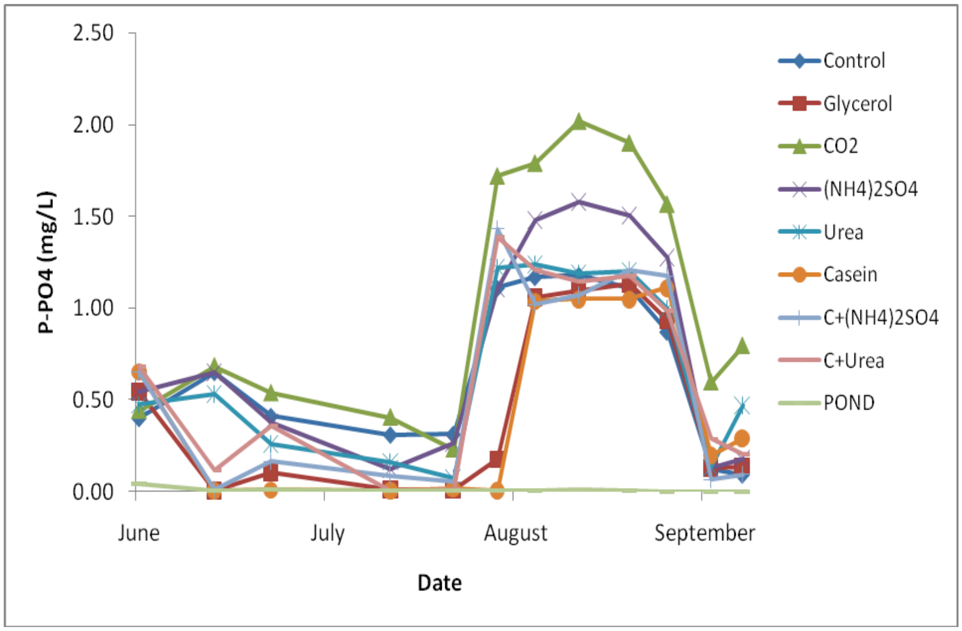

Figure 9. Phosphate concentration in different treatments. The solubility of phosphorus in carbon dioxide treatment was higher due to the lower of pH.

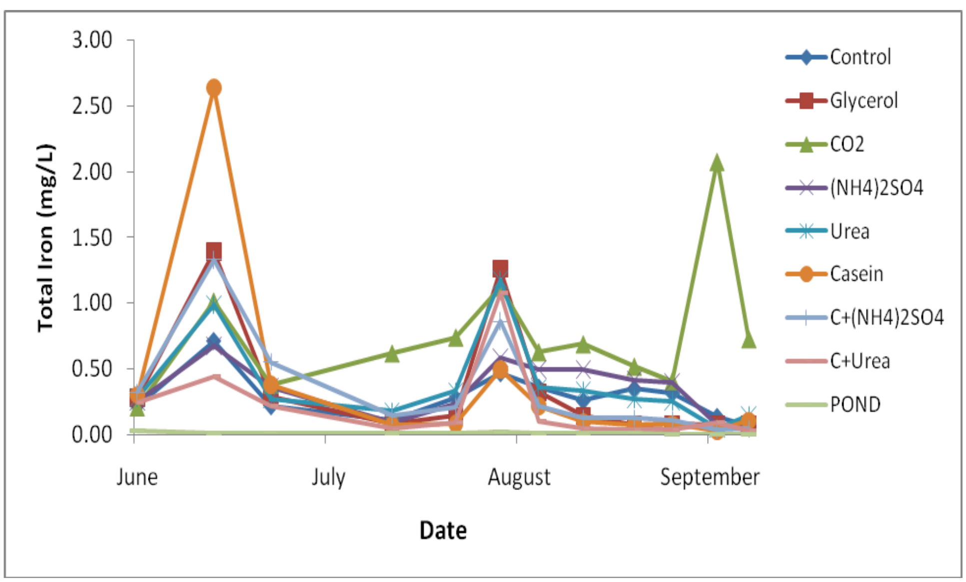

Figure 10. Total iron concentration in different treatments. The solubility of iron in carbon dioxide treatment was higher due to the lower pH.

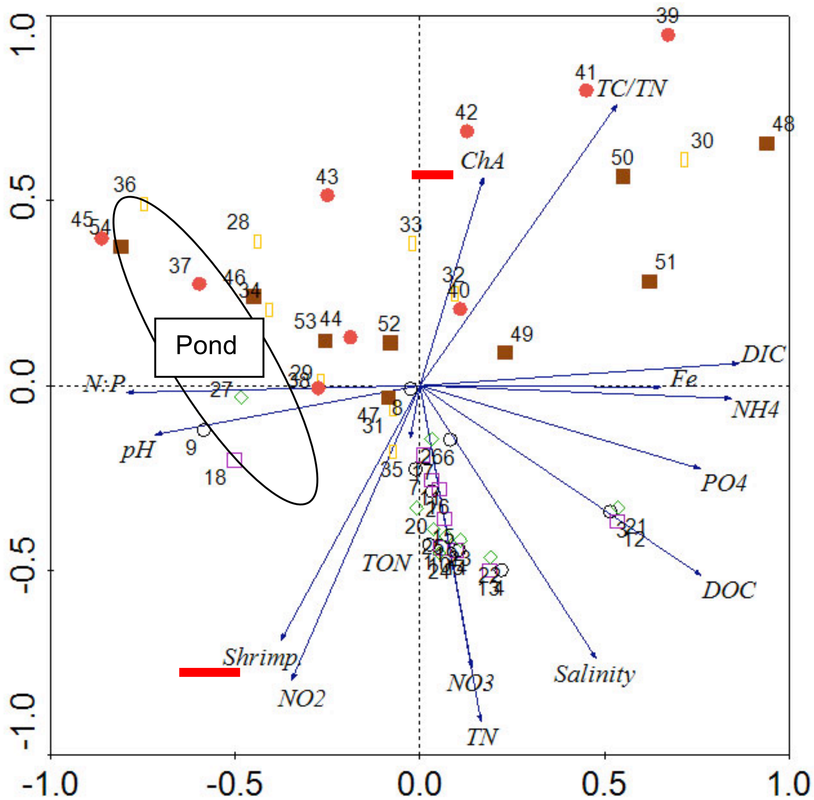

Figure 11. Multivariate analysis of different treatments in mesocosms and the pond (analyzed by CANOCO). Different colors show different sampling date. Water chemistry of the pond is different from the mesocosms. Shrimp numbers are correlated with nitrite concentration and pH, whereas the algal biomass as chlorophyll A (Ch A) is correlated with total dissolved carbon: nitrogen ratio and dissolved inorganic carbon.

The effect of different nutrient additions on water chemistry, algal biomass and shrimp production (numbers per L) was analyzed by a multivariate statistical approach using CANOCO. An ordination method was used for data clustering. The ordination of the samples was characterized by value on multiple variables (water parameters). Therefore similar samples were near each other and dissimilar samples were farther from each other. The arrows show the parameters in this experiment; the closer the arrows, the more positive correlation between the parameters.

The multivariate analysis of water chemistry, algal biomass, and shrimp number showed that the water chemistry has a greater correlation with the sampling time, (samples grouped closely in correlation with time as a vector). The pond samples could be separated from the mesocosms samples based on the different water chemistry (Figure 11). In addition, the multivariate analysis indicates that the shrimp numbers were correlated with nitrite concentration and pH, whereas the algal biomass is correlated with total dissolved carbon: nitrogen ratio and dissolved inorganic carbon.

In addition, we compared microalgal and microbial communities in different culturing. The results from light microscopy and DNA based methods show that the algal and microbial communities in mesocosms were similar to the communities in the pond. The communities in the microcosms incubated in the lab were very different. Figure 12 shows that green filamentous eukaryotic algae and diatoms dominated mesocosms, whereas the communities in the lab microcosms were more diverse and cyanobateria and microalgae were easily detected.

Denaturing gradient gel electrophoresis (DGGE) is a method in microbial ecology to separate PCR products based on their sequence (e.g GC content). DGGE banding patterns (PCR fingerprinting) can be used to visualize variations in microbial genetic diversity and provide a rough estimate of the richness and abundance of predominant microbial community members. In this experiment, PCR amplification with PCR primers specific for bacteria including cyanobacteria (microalgae) were used to obtain PCR fingerprinting of microbial communities in different field and lab experiments. Based on the observed PCR fingerprinting, the communities in the pond and mesocosms were very similar in comparison with microbial community fin the lab microcosms (Figure 13).

The dominant bands on the DGGE gel were analyzed by sequencing. Sequencing data showed that mesocosms and pond communities were dominated by *Amphora* sp. (diatom) whereas the microcosms were dominated by *Oscilatoria* sp. (filamentous cyanobacteria).

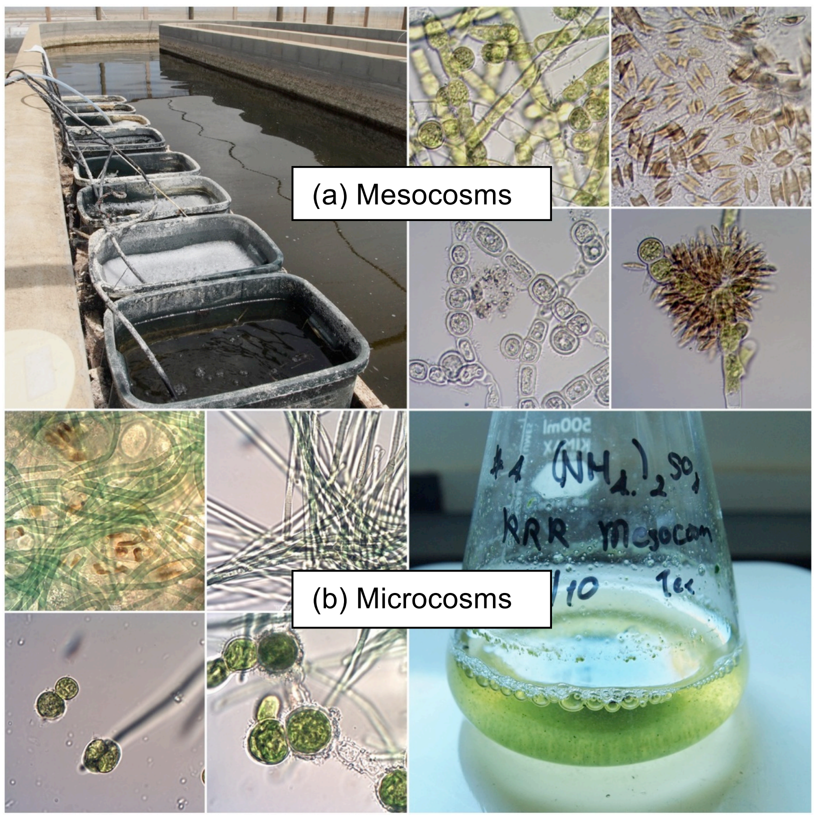

Figure 12. Microscopy of algal communities. (a) Algal communities in mesocosms were dominated by filamentous eukaryotic algae and diatoms, and (b) Algal communities in lab microcosms included small filamentous algae (cyanobacteria), diatoms, eukaryotic filamentous algae, and our isolated strain (RRR sp1).

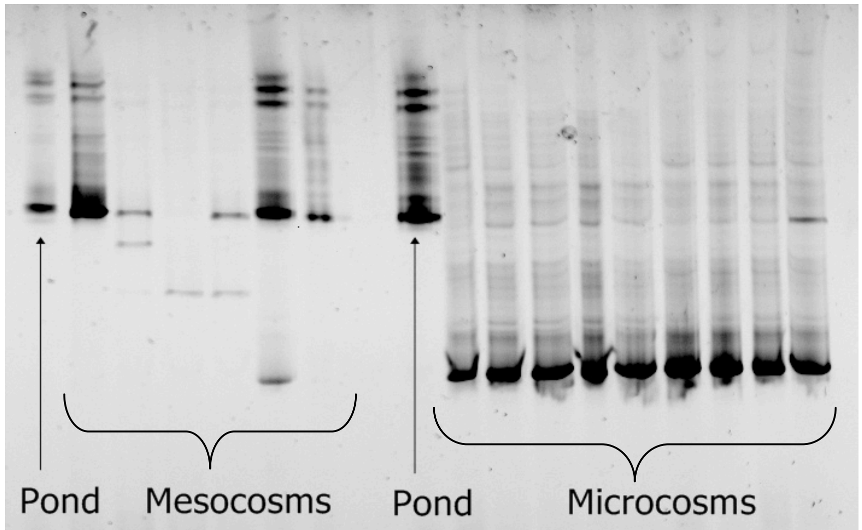

Figure 13. Denaturing gradient gel electrophoresis (DGGE) of microalgal and bacterial communities. Mesocosm communities were more similar to the pond community than to lab microcosms based on the observed PCR fingerprints and sequencing results.

III. Optimization of Culture Conditions of Alga Isolated from Red Rock Ranch Water

Background

The recent decade witnessed a surge of interest in algal lipids as a promising supplemental oil source for biodiesel production. Microalgal biomass production offers the potential to reduce greenhouse gas emissions by providing biofuel replacement of fossil fuels as well as carbon-neutral animal feeds. Although algae can grow in varieties of water from fresh water, seawater, to wastewater from plants or farm, RRR drainage water limits growth of many algal species due to the presence of high Se concentrations.

Objectives

Culturing native algae that grow in RRR water suitable for biomass production than introducing new strains, which cannot survive in contaminated water with high selenium and salinity level. The objectives of this experiment are to isolate algae from RRR water and optimize the culture condition for an increased biomass production. Mainly, we focused on:

1) Isolation and identification algae from RRR water
2) Optimization of culture conditions, salinity, photoperiod, nutrients for algal biomass production
3) Testing isolated algal strain suitability for brine shrimp production on a mesocosm scale

Isolation of native pure microalgal culture

Clonal cultures were established by streaking diluted water samples on 1% agar medium containing 50% of 60 ppth Na_2SO_4 solution and 50% of filtered sterile RRR water. Cultures were incubated at 25^oC under 24 hours light exposure. Single colonies were picked and transferred to 5 ml of filtered sterile RRR water without agar in 10 mm capped glass test tubes. Cultures were established by serial re-streaking on agar plates and isolating of single colonies. The absence of bacteria was established by streaking on nutrient media and careful observation under a microscope.

DNA extraction, amplification and sequencing
Algal cells were harvested from growing culture by filtering through 0.22 μm GTTP filter. Filters were stored at -20 ^{o}C freezer. DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega). Polymerase Chain Reaction (PCR) (Techne, thermocycler) amplification of a portion of the 16S rRNA gene was performed with specific primers CYA781R (a) and CYA359F. The purified PCR products were submitted to the College of Biological Sciences Sequencing Facility (Davis, California) for sequencing. Phylogenetic assignments of the sequences were determined using Basic Local Alignment Search Tool (BLAST).

Pure green algal strain isolated from RRR water
Picocystis salinarum RRR1, a unicellular marine green alga belonging to the phylum *Chlorophyta* (Lewin et al 2001) was successfully isolated and purified. The taxonomic identity of the strain was based on 16S rRNA sequencing. This strain has spherical cells, about 2-3 μm in diameter. For laboratory experiments the strain was maintained in 500 mL flasks containing 200 mL RRR water.

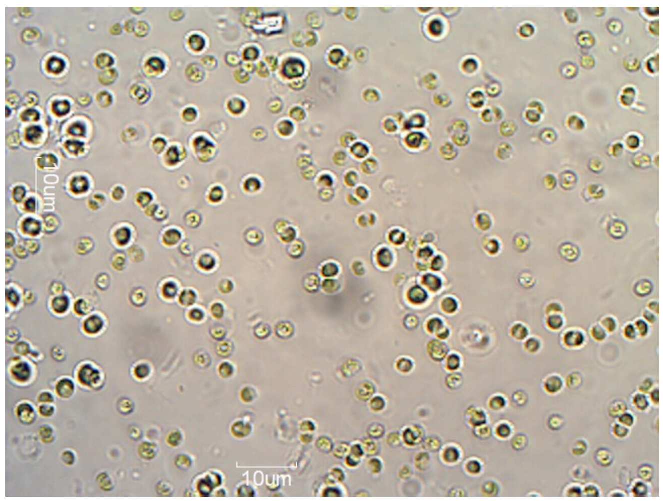

Figure 14. Phase contrast micrograph of *Picocystis salinarum* RRR1

Strain optimization for biomass production

a. Growth curve and biomass conversion

To determine the biomass production, the experiment was set by measuring optical density (OD_{685}) and dry weight. The results show that the OD_{685} and dry weight have a linear correlation; y=455.22x+46.017; $R^2 = 0.959$.

From this equation, we use factor 455 * OD_{685} to estimate algal biomass.

b. Salinity

The experiment was designed to determine whether different salinity has affect on the algal growth. The growth media was prepared by mixing RRR water with Na_2SO_4 solution to adjust salinity. There were 4 treatments with different salinities 40, 60, 120, and 150 ppth.

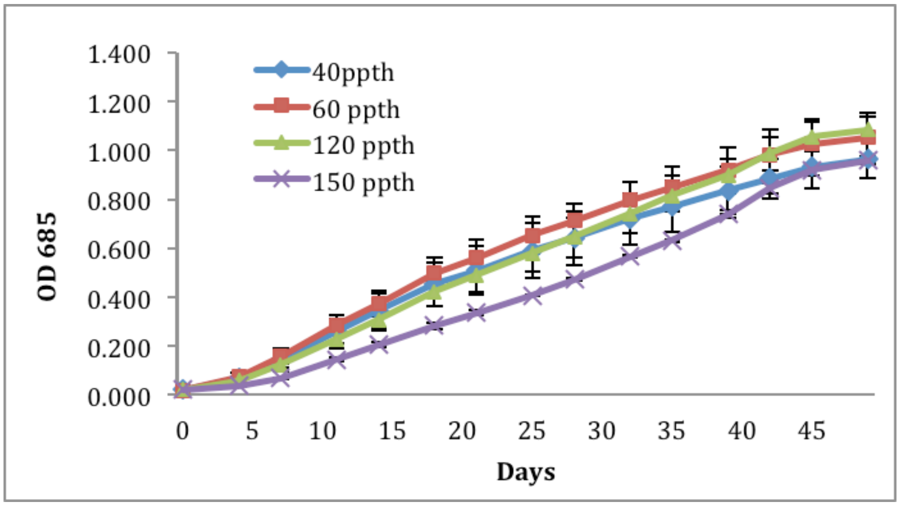

Figure 15. Growth of *Picocystis* strain in RRR water with different salinities

The results show that this green alga grew under all experimentally tested salinities and is tolerant to extremely high salt concentrations.

c. Photoperiod

An experiment was conducted to determine the optimal growth condition under different light regime. The experiment was set up with three different treatments; 12, 16, and 24 light hours.

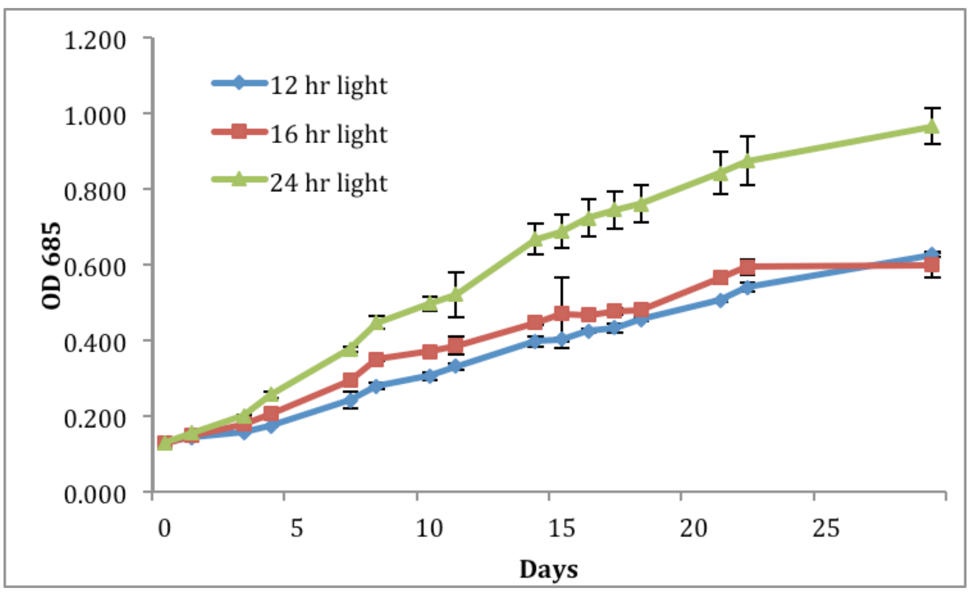

Figure 16. Growth of *Picocystis* sp. at different photoperiod

The results showed that 24 hour light regime stimulated significantly higher algal growth and biomass production. *Picocystis* sp. doubling time in 24-hour light regime was 8 days. The strain doubling times were 11 days and 10 days at the 12- and 16-hour light treatments respectively.

d. Iron

Iron deficiency is known as limiting factor for algal biomass productivity. RRR water has very low soluble iron. Iron might be one of the key factors in enhancing algal biomass production in RRR drainage water. The experiment was set up under batch culture condition with 4 different concentrations of Fe (II) gluconate; 0,5,10, and 20 ppm.

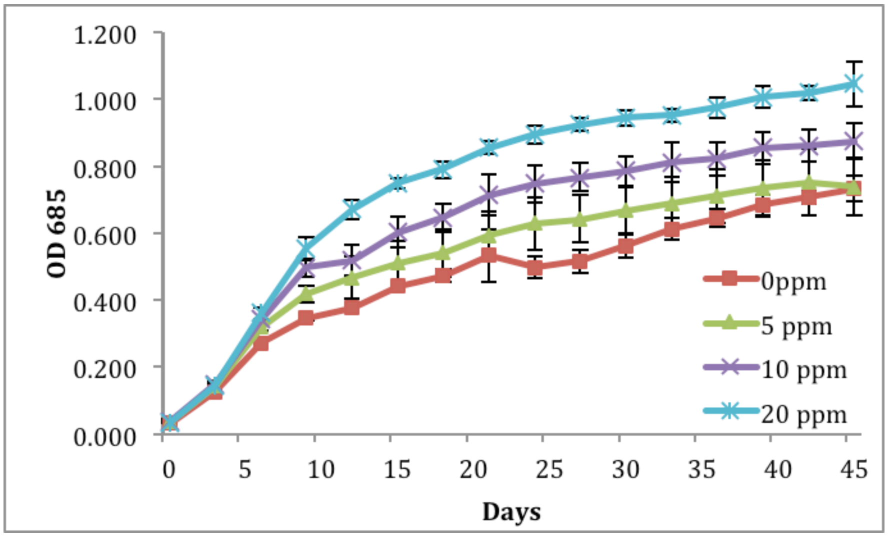

Figure 17. Growth of *Picocystis* sp. in medium with different iron addition

The results show that the higher concentration of Fe(II) gluconate, the higher algal biomass production. Moreover in the 20 ppm treatment, *Picocystis* sp. had higher growth rate and reached a stationary phase after 15 days of incubation. Doubling time of the strain was 3.36 days compared to 8 days in media without Fe addition. Based on this result, *Picocystis* sp. was grown in RRR water with 20 ppm Fe(II) gluconate addition for regular strain maintenance in the lab.

e. Carbon sources

Addition of organic carbon source is widely used in heterotrophic algal culturing and based on the strain physiology might produce higher algal biomass in comparison to phototrophic algal growth. Previously we determined that *Chlorella* sp. UTEX 2341 growth was improved with addition of an organic carbon source in 2L batch reactor. Algal biomass and lipid production increased with glycerin as carbon source, and the initial concentration of glycerin was the most significant factor for the increase (Yang et al., 2011). Similarly, an experiment was designed to investigate the effects of different organic carbon sources such as glycerol, starch, sodium acetate, and glycine, on *Picocystis* sp. growth. The carbon moles of additives were calculated to 0.1 M glycerol, except for the starch.

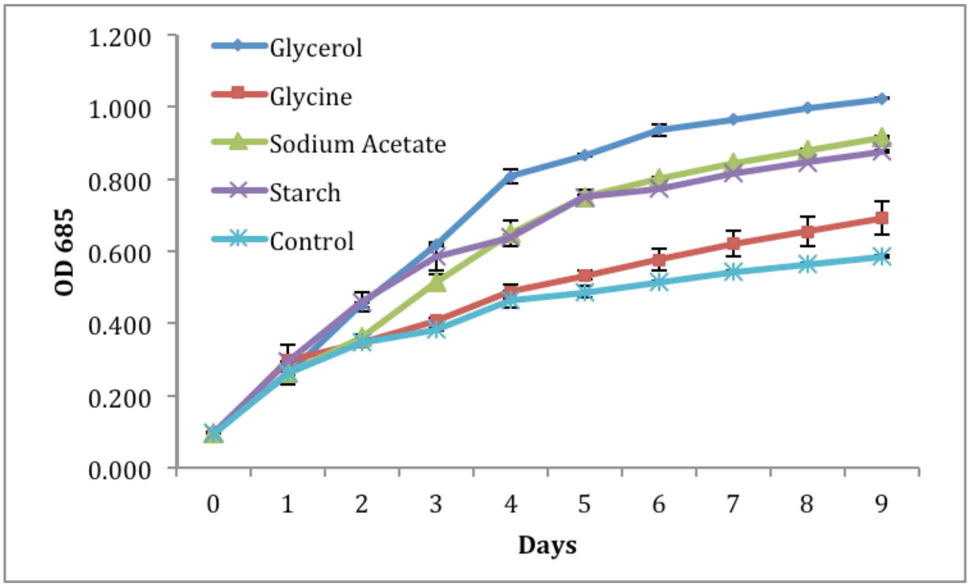

Figure 18. Growth of *Picocystis* sp. in media with different carbon source addition.

The results show that carbon addition into culture media increased biomass production of *Picocystis* sp. The highest biomass production was found in the media with glycerol addition and culture growth was improved based on the observed doubling time of 2.66 days. The results indicate that *Picosystis* sp. is a mixotroph that is able to utilize inorganic and organic carbon as C-source and thus has a potential to be optimized for lipid production.

Mesocosm Experiment

The experiment was set up to determine whether *Picocystis sp.* is a suitable brine shrimp food and if it's addition will enhance brine shrimp production. Four 100L plastic tubs were used as containers and they were separate into two sets; 2 with algae addition and 2 with no algae addition (Figure 19). In all tubs, water from Red Rock Ranch was added with native algae and original brine shrimp population. Bubbling system was used for mixing and aeration, and carbon dioxide gas was added in order to increase algae production. A pH regulator at pH 7 regulated the amount of carbon dioxide flow. Algal inoculum, grown in the laboratory, was added into 2 of the tubs to build up the *Picocystis sp.* algal population. The algae addition continued for 2 months until the algae population reached steady state or increased without further addition. Water chemistry, algal population and brine shrimp biomass were monitored.

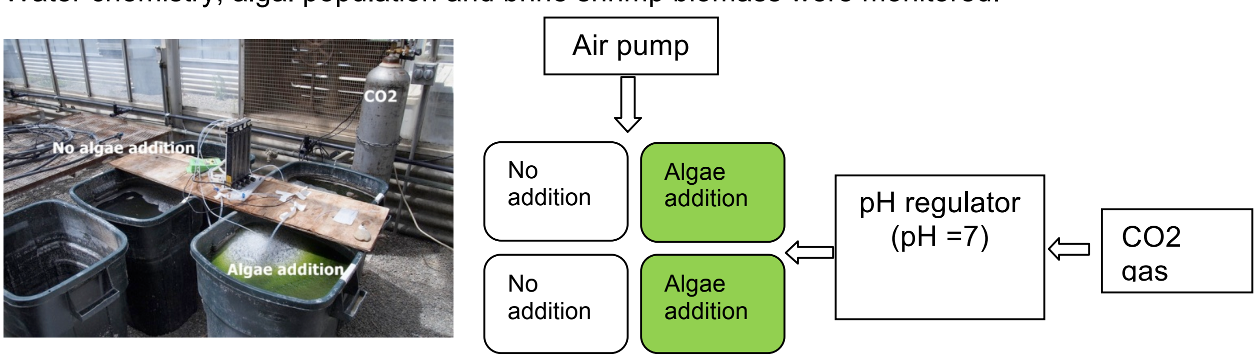

Figure 19. Mesocosm experiment set up in a green house under controlled temperature.

Mesocosm Results

After adding algae into the mesocosms, algal biomass (measured as chlorophyll) increased continuously from February through May (Figure 20) in comparison with the control mesocosms where the algal biomass was stable throughout the experiment. The results indicate that *Picocystis sp.* can grow well in the mesocosm system. The algal biomass was increasing even after we stopped addition because the amount of added algae was more than the consumption by brine shrimp in the mesocosms.

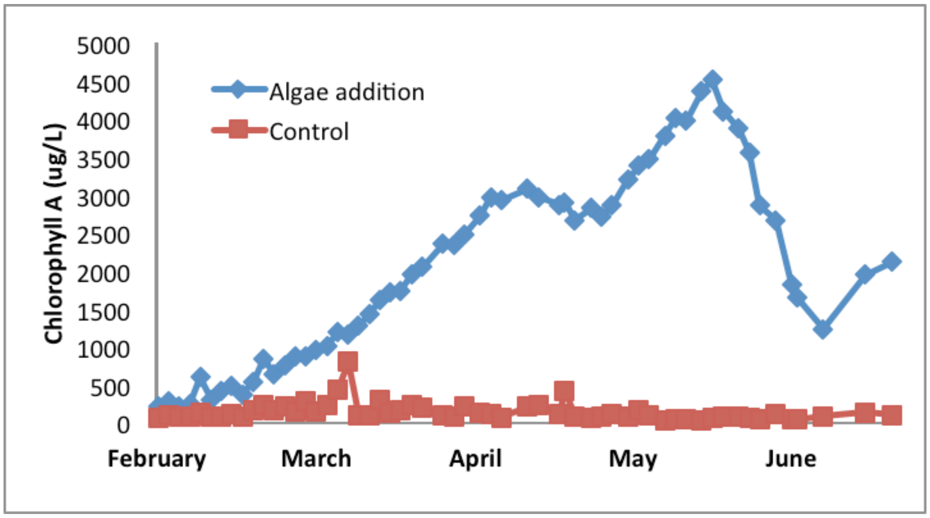

Figure 20. Algal biomass, measured as chlorophyll, in mesocosm incubations

a) b)

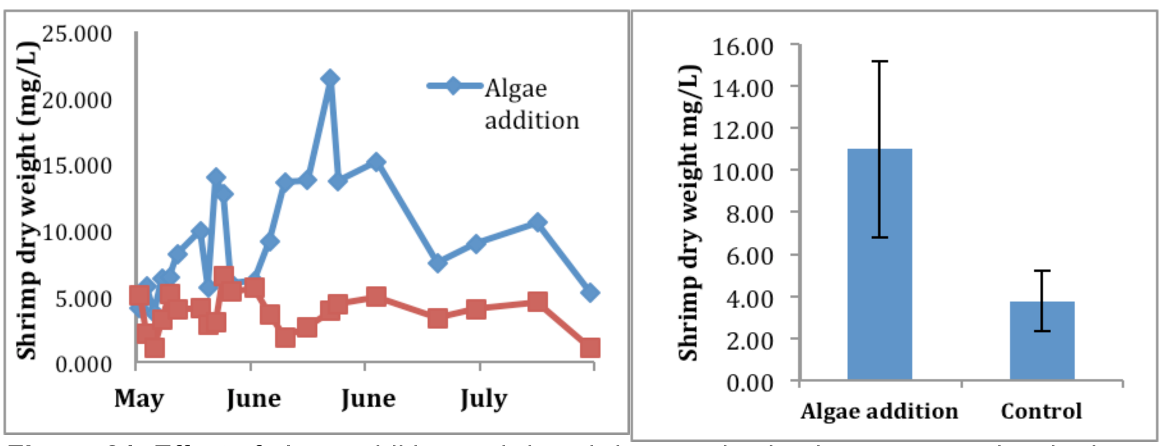

Figure 21. Effect of algae addition on brine shrimp production in mesocosm incubations

During mid-May to June, the algal biomass decrease correlates with the observed increase in brine shrimp production (Figure 21 a) most likely due to grazing. The observed increase in brine shrimp production was significantly higher in the mesocosms with algae addition (Figure 21 b) compared to the control mesocosms.

IV. The biochemical transformation of selenium inside aquatic organisms living in the RRR pond

To evaluate the ecotoxic status of the engineered algae-brine shrimp ecosystem we collaborated with Dr. John Freeman (Department of Biology, California State University, Fresno, CA) and Dr. Gary Bañuelos (Agricultural Research Service, United States Department of Agriculture, Parlier, CA). We fulfilled this objective by using a variety of controlled growth and culture systems together with a combination of analytical tools, including micro focused X-ray fluorescence (mXRF) elemental mapping, Se K- edge X-ray absorption near edge structure (mXANES) spectroscopy and strong anion exchange high performance chromatography coupled to real time inductively coupled plasma mass spectrometry (SAX-HPLC-ICPMS). Bacteria, algae, diatoms, and brine shrimp were cultivated on regular basis in the laboratory of Dr. Hristova at UC Davis and in the RRR cultivation pond, where an engineered algae-brine shrimp ecosystem was established. Dr. Freeman led the effort of applying advanced analytical methods to understand Se biochemical transformations in this ecosystem. We have been also fortunate to work with Drs. Sirine Fakra, Matthew Marcus, Soo In Yang, and Ingrid Pickering and have access to the analytical capabilities in the Advanced Light Source, Lawrence Berkeley National Laboratory, CA, and the Department of Geological Sciences, University of Saskatchewan, Canada.

The characterization of Se bio-accumulation and bio-transformation by *Artemia* and *Ephydra* (and organisms they feed upon) is environmentally important in these wetland environments, because both are the major food sources for vast numbers of breeding and migratory birds. The understanding of how Se-biotransformation takes place moving up the food chain provides insight for managing Se-threatened aquatic ecosystems and for producing Se-enriched brine shrimps used in farm animal feed.

Methods
In the engineered aquatic pond system the biological fate of Se was determined, through analyzing Se-accumulation and Se-biotransformation within bacteria, diatoms, and microalgae (*Picocystis* sp.), macroalgae (*Chlorophyta*), the macroinvertebrates brine fly (*Ephydra cinerea*) and brine shrimp (*Artemia franciscana*). We achieved this by using a variety of controlled growth and culture systems together with a combination of analytical tools such as micro focused X-ray fluorescence (µXRF) elemental mapping, Se K- edge X-ray absorption near edge structure (µXANES) spectroscopy and strong anion exchange high performance chromatography coupled to real time inductively coupled plasma mass spectrometry (SAX-HPLC-ICPMS) (*detailed description of the methods in Appendix*).

Results
Accumulation, Localization and Speciation of Se in Aquatic Organisms

A suite of complimentary analyses was used in conjunction with both laboratory and field experiments to gain insight into the chemical speciation, localization and accumulation of Se in the aquatic organisms thriving in the RRR drainage water engineered aquatic ecosystem. The relevance of our findings are furthermore extrapolated into the analogous, naturally-occurring brine wetlands that are commonly threatened by irrigation runoff containing Se originating from vast agricultural areas such as those located in the WSJV and western slope of Colorado.

The chromatographic separation of the soluble-Se forms in the undigested, and proteinase K digested, aqueous extracts of RRR water grown mixed bacteria, mixed diatoms, pure green microalga strain *Picocystis* sp., brine fly *Ephydra cinera*, and brine shrimp *A. franciscana* was achieved via SAX-HPLC-ICPMS, and resulted in the determination and quantification of multiple ^{78}Se-containing peaks for each sample (Fig. 22). Using μXRF mapping, the spatial distribution of Se was mapped in flash-frozen samples of mixed bacteria and diatoms, and in a pure culture of microalgae (*Picocystis* sp.) as shown in Fig. 23 and 24. All the microflora exhibited a uniform distribution of Se except in the mixed bacterial culture, which showed Se-enriched spots, likely bacterial deposits, subsequently determined to be primarily elemental Se^0 using μXANES analysis.

The spatial distribution of Se, Ca and Zn in the brine fly *Ephydra cinera*, as well as the brine shrimp *A. franciscana,* invertebrates that consume the above discussed microbes in the RRR pond system is shown in Fig. 23 and Fig. 24. Based on the maps we then examined the chemical forms of Se at the microscopic level in the most Se-concentrated areas of these samples using μXANES and these results are presented in (Table 4). Furthermore, we employed bulk XANES to estimate the mean Se speciation averaged over the whole ground tissue of each sample. Because whole organisms were sampled, bulk XANES complemented the SAX-HPLC-ICPMS of the soluble-Se forms and helped guide μXANES analyses.

Bacteria

The predominant forms of Se resulting from bacterial biotransormations depend on environmnental conditions, as well as bacterial species present. In the bacterial samples grown in RRR drainage water, SeO_4^{2-} predominated, with only minor conversion to organic Se forms (Figure 22 A, Tables 2,3). The average total Se for the bacterial sample was 2.5 ± 0.4 μg g^{-1} WW (wet weight). The percentage of Se recovery into the aqueous phase for the proteinase XIV-digested sample was 84.1%, and proteinase XIV treatment increased soluble-Se recovery by 1.2 fold.. Numerous Se bright spots were observed in the mixed bacteria and diatom culture by μXRF. Further examination of these areas by μXANES revealed they consisted of elemental Se (Se^0) (Table 4), which is consistent with dissimilatory SeO_4^{2-}, SeO_3^{2-}, Se^{2-}, reduction into the elemental form by bacteria. Although methylation and subsequent volatilization of Se are thought to constitute significant pathways for Se removal from contaminated systems, little evidence for volatile forms or their immediate precursors were observed in these samples.

Diatoms

An axenic culture of mixed diatoms was isolated as described above. Culture purity was assessed by microscopy and heterotrophic plate counts. Cultures with no observed bacterial or other microalgal contamination were used for further study. Mixed diatom cultures grew well in RRR water even at the highest Se concentration tested (Se 40 mgL^{-1}). Although thick silica cell walls render diatoms difficult to digest, they are grazed by *Artemia* (Mohebbi 2010) and are a preferred food source for *E. cinera* larvae (Wurtsbaugh 2007). In addition, diatoms are the principal food of deposit-feeding bivalves such as *Macoma balthica* that live in San Francisco Bay (Luoma et al. 1992).

SeCyst and CysSe-SeCys, and to a lesser degree SeMet were significant in the diatom samples but SeO_3^{2-} also accounted for a significant portion of cell-associated Se (Tables 1, 2) by SAX-HPLC-ICPMS. The average total Se for the diatom sample was 59.8 ± 1.9. μg g^{-1} WW. The percentage of Se recovery into the aqueous phase for the proteinase XIV-digested sample was 17.7%, and proteinase XIV treatment increased soluble-Se recovery by 1.9 fold. The 23.7% SeO_3^{2-} was the highest percentage of Se measured in all organic samples and was about 7 times higher than amount of SeO_4^{2-} measured in the diatom sample. Similarly, a high 6:1 SeO_3^{2-} to SeO_4^{2-} ratio was also determined in the μXANES analysis, although no organic Se forms were observed (Table 4). Mixed diatoms showed relatively uniform Se distribution by μXRF mapping (Figure 23-B). Since there was little SeO_3^{2-} present in the drainage water circulating in the RRR pond system, internal diatom SeO_4^{2-} reduction presumably led to the high internal SeO_3^{2-} concentrations. Selenate reduction could be a detoxification measure. Selenium is an essential nutrient for some freshwater and marine diatoms (Araie et al. 2009; Harrison et al. 1988; Price et al. 1987). Unlike higher plants, some diatoms rely on selenoproteins such as selenoprotein glutathione peroxidase (used for oxygen detoxification) (Raven et al. 1999), and may therefore require a high affinity Se uptake mechanism.

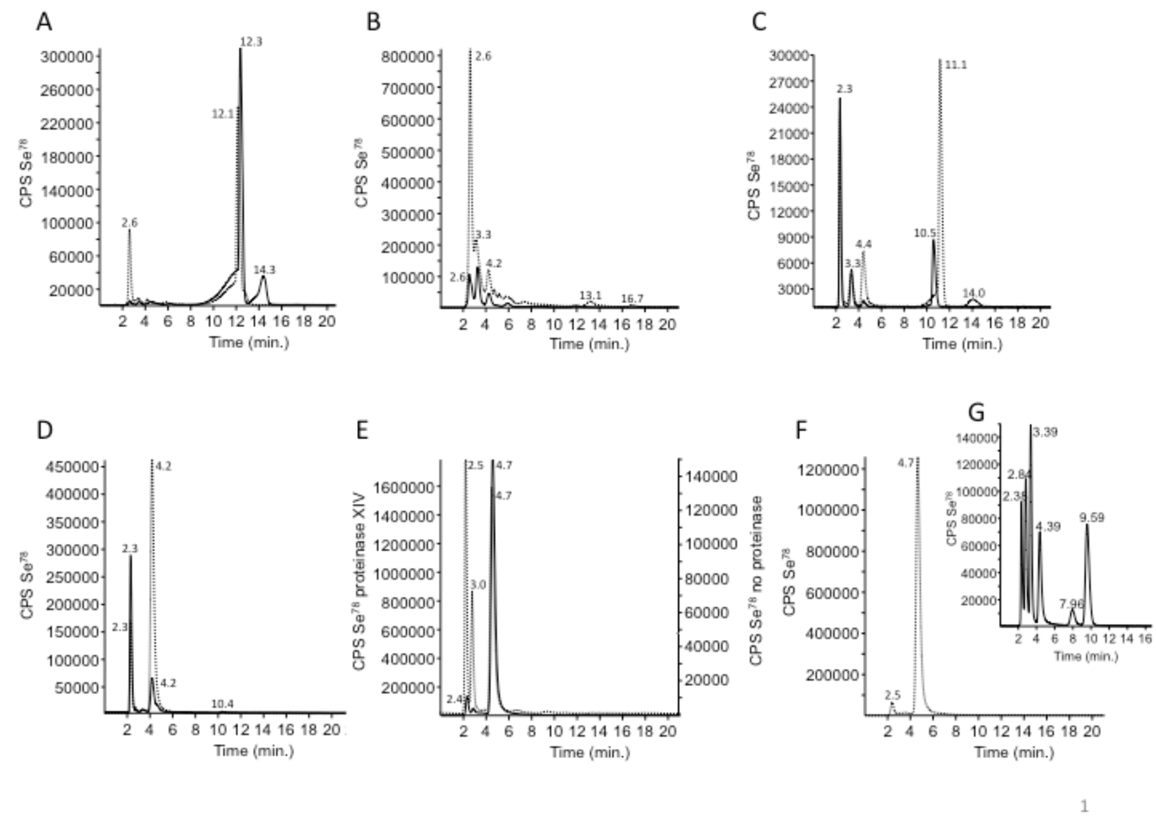

Figure 22. Chromatographic separation and identification of the soluble Se compounds present in members of the RRR water food web before and after proteinase XIV-digestion using strong anion exchange high pressure liquid chromatographic inductively coupled mass spectrometry (SAX-HPLC-ICPMS). Selenium containing peaks, monitored as Se^{78}, are shown as mixed bacteria (A); mixed diatoms (preliminary) (B); *Picocystis* sp. - pure microalga (C); *A. franciscana* (D); Ephydra larva (E); Ephydra adult (F), standards (G). Undigested (solid line) and proteinase XIV-digests (dashed line) chromatographs of organisms from the RRR water system. Sample Se containing peak retention times were compared to those of the authentic Se standards (G). Peaks are quantified as percentage of total detectable soluble Se^{78} in Table 2.

Green picoalga (*Picocystis* sp.)

The unicellular green picoalga *Picocystis* sp. (Lewin et al. 2000) was isolated from RRR water (Prapakorn, manuscript in preparation). While the contribution of this strain to the primary production in the RRR pond was not determined, *Picocystis* species can be significant primary producers. In Mono Lake, CA, *Picocystis* sp. strain ML4 accounts for 25% to 50% of primary production at various times of the year, and is heavily grazed by the endemic brine shrimp *Artemia monica* (Roesler et al. 2002). Distribution of Se in *Picocystis* sp. at RRR was roughly half organic and half a mixture of SeO_4^{2-} and SeO_3^{2-} (Tables 2, 3). The organic portion was dominated by SeCyst. The average total Se for the *Picocystis* sp. sample was 1.2 ± 0.7 μg g^{-1} WW. The percentage of Se recovery into the aqueous phase for the proteinase XIV-digested sample was 16.4%, and proteinase XIV treatment increased soluble-Se recovery by 4.1 fold. The proportion of SeO_4^{2-} in *Picocystis* sp. increased significantly in the proteinase XIV-digested samples (Tables 2, 3). *Picocystis* sp. showed relatively uniform Se distribution by μXRF mapping. Se concentration in *Picocystis* sp. culture was below the detection limit for μXANES analyses. In higher plants, SeO_4^{2-} and SeO_3^{2-} are reduced to Se^{2-} by glutathione before incorporation in selenoproteins, with uptake and assimilation by a route similar to the sulphur assimilatory pathway (Fournier et al. 2010; Hamilton 2004; Pyrzynska 2002). The transport of Se into green algae is likely due to high affinity mechanisms at low concentrations and low affinity mechanisms with poor discrimination between Se species (such as SeO_3^{2-} and SeO_4^{2-}) at high Se concentrations.

Brine Fly (*Ephydra cinera*)

The entire *E. cinera* lifecycle is associated with brine lakes. *E. cinerea* thrive in environments with higher salt concentrations than other *Ephydrids* (Rosetta et al. 1995). Adult *E. cinerea* deposit sticky eggs on the water surface. At room temperature, hatching requires 5-6 days, larval development 18-206 days (depending on food supply), and the pupal stage about 15 days (Collins 1980). Adult flies usually live less than a week. Larvae feed on epilithic algae, and show a preference for diatoms (Wurtsbaugh 2007). Adults have limited opportunities to feed, but they will feed on epilithic algae and detritus just below water surface. Based on feeding patterns, it is clear that the majority of Se in adults is accumulated during larval stage. In this study, the Se species profile in larva and adult *E. cinera* was significantly different from the Se profiles of the diatoms, bacteria and microalgae that typically constitute their diet (Tables 2, 3). The average total Se for the *E. cinera* larva sample was 59.1 ± 3.1 μg g^{-1} WW. The percentage of Se recovery into the aqueous phase for the proteinase XIV-digested sample was 73.5%, and proteinase XIV treatment increased soluble-Se recovery by 7.6 fold. Only the proteinase XIV-digested *E. cinera* adult sample was processed. The total Se for the *E. cinera* adult was 168.8 μg g^{-1} WW, with Se recovery of 77.8%. Essentially all Se in the *E. cinera* samples was detected in organic carbon-Se-carbon (C-Se-C) forms similar to SeMet or SeCyst (Table 4). These results were in good agreement with the SAX-HPLC-ICPMS analysis (Figure 22-E, F; Tables 2,3). This is in contrast to the prey-species samples which contain 33% - 75% SeO_4^{2-} or SeO_3^{2-}. A recent study found a thousand-fold bioconcentration between Se in the water columns and in the periphyton (surface organisms) in the Great Salt Lake in Utah, although further data suggested that there was no further bioconcentration between the periphyton and the brine flies (Wurtsbaugh 2007). In another study, biomagnification was determined to be the primary Se uptake pathway by *E cinerea* larvae tested in a 48-h bioassay with SeO_4^{2-}, SeO_3^{2-} and SeMet at concentrations ranging from 10-20 g L^{-1} (Rosetta et al. 1995).

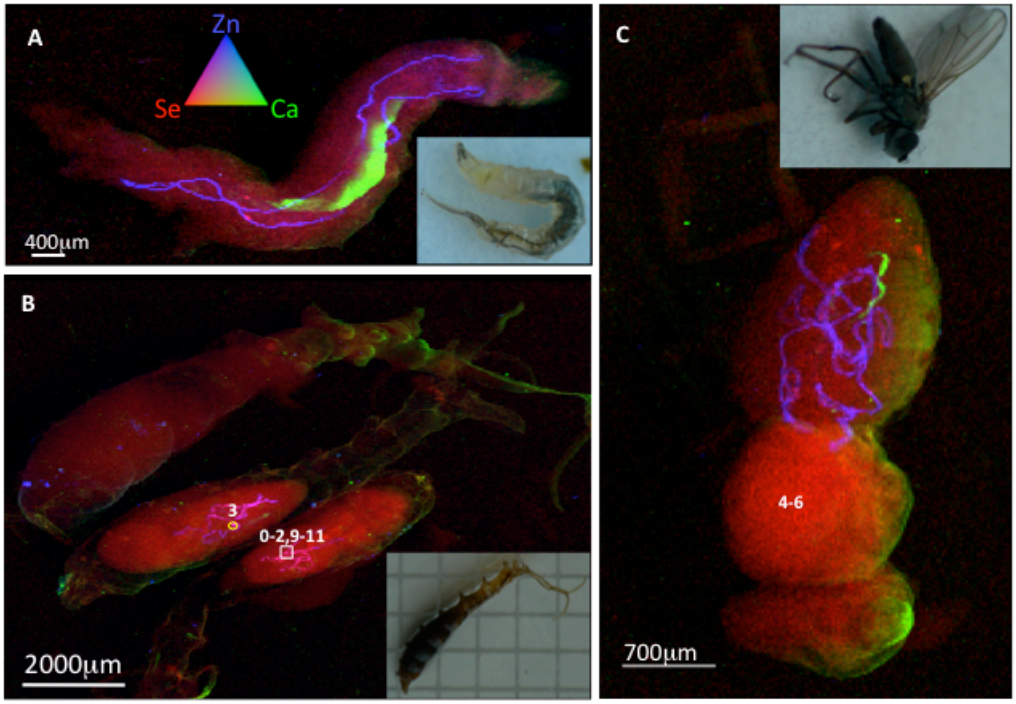

Figure 23. Distribution and speciation of Se in the brine fly *Ephydra cinera*. µXRF map showing spatial distribution of Se (coded in red) and other selected elements (calcium [Ca] in green, zinc [Zn] in blue) in fly larva (A), three pupae (B) and adult fly (C). Circles in A, B and C show locations of µXANES scans. Light micrographs of similar individuals are shown as inserts.

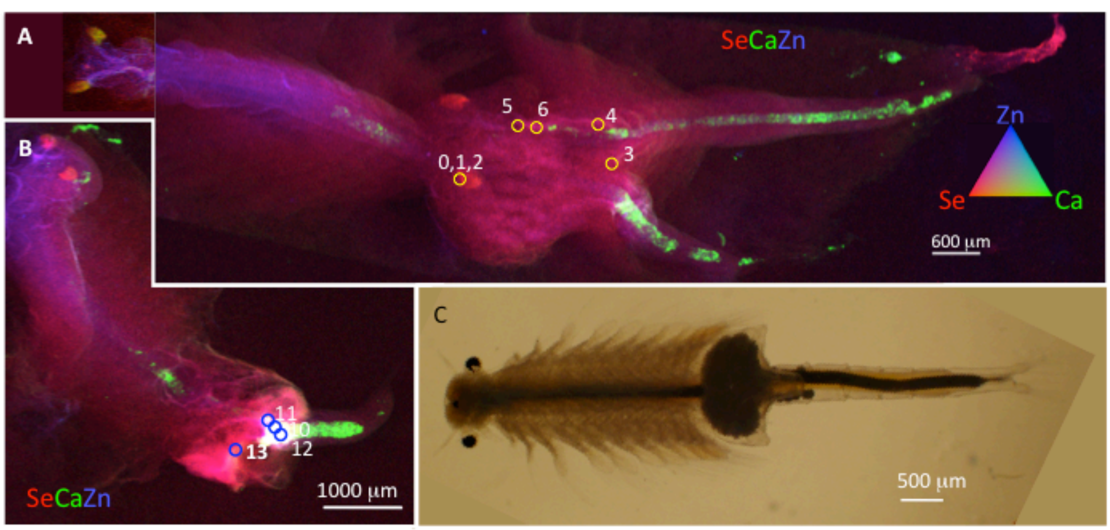

Figure 24. Distribution and speciation of Se in *Artemia franciscana*. µXRF map showing spatial distribution of Se (coded in red) and other selected elements (calcium [Ca] in green, zinc [Zn] in blue) in adult female with egg sac and adult male, coupled (A), in single adult female with egg sac (B). Adult female with egg sac shown for comparison (C). Yellow circles in A and B show locations of µXANES scans.

Table 2. SAX-HPLC-ICPMS speciation of soluble Se extracted from ground, undigested tissues.

No proteinase XIV treatment	bacteria		bacteria		bact/diatom		bact/diatom		Picocystis sp.		Picocystis sp.		Ephydra larva		A. franciscana		MW7 standard		
[Se] in growth media (mg/L)	40		40		40		~9		40		~9		~9		~9		~9		
Se Species	RT (min	Se (%)	RT (min	Se (%)	RT (min	Se (%)	RT (min	Se (%)	RT (min	Se (%)	RT (min	Se (%)	RT (min	Se (%)	RT (min	Se (%)	RT (min	Se (%)	RT average
SeCyst or CysSe-SeCys	2.6	0.7%	2.6	0.7%	2.4	0.5%	2.6	32.8%	2.3	51.2%	2.3	33.5%	2.4	30.2%	2.3	63.4%	2.4	11.7%	2.5 ± 0.1
MeSeCys					2.8	4.8%							2.9	16.6%			2.8	17.0%	2.8 ± 0.1
SeO_3^{2-}	3.5	0.7%	3.3	1.3%	3.3	2.1%	3.3	41.2%	3.3	14.8%	3.3	8.0%			3.3	2.9%	3.4	23.3%	3.3 ± 0.1
SeMet	4.5	1.9%	4.3	2.2%	4.2	4.9%	4.3	16.7%	4.4	2.7%	4.4	5.2%	4.7	53.1%	4.2	33.1%	4.4	19.0%	4.3 ± 0.1
CysSe-SeCys	6.1	0.5%	5.7	0.5%			6	4.5%											5.8 ± 0.2
gGMeSeCys							8	2.7%									8	4.4%	7.8 ± 0.3
SeO_4^{2-}	12.3	82.5%	12.2	83.3%	12.4	74.9%	11.9	2.1%	10.5	31.3%	11	53.3%			10.5	0.5%	9.6	24.7%	11 ± 1
Seleno Met-Protein	14.3	13.7%	13.6	12.0%	14.1	12.7%													14 ± 1.3

Table 3. SAX-HPLC-ICPMS speciation of soluble Se extracted from ground, protease XIV-digested tissues.

No proteinase XIV treatment	bacteria		bacteria		bact/diatom		bact/diatom		Picocystis sp.		Picocystis sp.		Ephydra larva		Ephydra adult		A. franciscana		MW7 standard		
[Se] in growth media (mg/L)	40		40		40		~9		40		~9		~9		~9		~9		~9		
Se Species	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT (min)	Se (%)	RT average
SeCyst or CysSe-SeCys	2.6	14.5%	2.6	11.3%	2.6	14.1%	2.6	45.2%	2.3	22.8%	2.3	21.0%	2.5	4.1%	2.5	3.7%	2.3	15.0%	2.4	11.7%	2.5 ± 0.1
MeSeCys													3.0	3.7%			2.7	1.3%	2.8	17.0%	2.8 ± 0.1
SeO_3^{2-}	3.4	2.7%	3.3	2.2%	3.4	3.4%	3.1	23.7%	3.3	8.5%	3.3	11.3%			3.7	2.1%	3.4	1.4%	3.4	23.3%	3.3 ± 0.1
SeMet	4.2	2.2%	4.2	1.7%	4.3	4.0%	4.2	11.0%	4.4	13.8%	4.4	32.0%	4.7	92.1%	4.7	94.2%	4.2	81.6%	4.4	19.0%	4.3 ± 0.1
?			4.7	0.6%	4.8	0.6%	4.7	3.0%													4.7 ± 0.0
?	4.9	0.5%					5.2	3.4%													5.1 ± 0.2
CysSe-SeCys	5.8	1.6%	5.6	1.1%			5.8	6.2%													5.8 ± 0.2
gGMeSeCys							7.4	3.7%											8.0	4.4%	7.8 ± 0.3
SeO_4^{2-}	12.1	75.2%	12.0	76.8%	11.2	77.9%	13.1	3.2%	11.1	51.1%	10.8	35.7%					10.4	0.6%	9.6	24.7%	11.4 ± 1.0
Seleno Met-Protein	12.7	3.3%	12.8	6.4%			16.7	0.5%	14.0	3.8%											14.0 ± 1.3

Brine Shrimp (*Artemia franciscana*)

Brine shrimp are filter feeders that feed on the mix of microalgae, diatoms and bacteria in the water column. The average total Se for the *A. franciscana* sample was 7.2 ± 0.6 μg g^{-1} WW. The percentage of Se recovery into the aqueous phase for the proteinase XIV-digested sample was 63.8%, and proteinase XIV treatment increased soluble-Se recovery by 3.9-fold. Micro-XRF mapping revealed that in adult brine shrimp (*A. franciscana*), the muscle along the back, eggs called cysts, and eyes (two stalked compound) accumulated Se (Figure 24). Similarly to *E. cinera*, Se species profile in adult *A. franciscana* harvested from the RRR pond was significantly different from the Se profiles of the phytoplankton (bacteria, diatoms, microalgae) that constitute their diet. In particular, *A. franciscana* had very little inorganic SeO_4^{2-} or SeO_3^{2-} as evidenced by SAX-HPLC-ICPMS (Fig 22 D; Tables 2,3) and µXANES (Table 4).

The response of *A. franciscana* to changing water Se concentrations was studied by analyzing total Se in *A. franciscana* harvested after controlled laboratory growth under varying Se concentrations. Freeze-dried *A. franciscana* samples analyzed by spectrofluorimetry showed proportional increase in internal Se concentration with the increase of the water Se concentration (Figure 25). The *A. franciscana* dry weight to wet weight ratio is approximately 1:10. When the measured dry weight Se concentrations were converted to calculated wet weight Se concentrations, the *A. franciscana* tissue Se concentration matched closely with the Se concentration in agricultural drainage water (Figure 25). This means that at an ambient concentration of 10 mg L^{-1} Se, *A. franciscana* harvest would contain approximately 10 g ton^{-1} Se. As *A. franciscana* accumulate bioavailable Se from their food (not water), the internal and environmental Se concentration equivalence must originate from an increase in organic Se in the primary producers at RRR.

Table 4. Se speciation in mixed bacterial culture, mixed diatom culture, *E. cinera* and *A. franciscana* tissue by µXANES spectroscopy Results of the least squares LCF of Se K-edge XANES spectra are shown. The best LCF was obtained by minimizing the normalized sum-squares residuals [NSS = 100 x $S(m_{exp} – m_{fit})^2/S(m_{exp})^2$], where m is the normalized absorbance. Error on percentages is estimated to ±10%. $SeGSH_2$, SeCys, SeCystine, and elemental Se^0 were not detected (empty boxes). Se^0 is both red and gray elemental forms.

Frozen tissue sample location	**NSS**	**Selenate (SeO_4^{2-})**	**Selenite (SeO_3^{2-})**	**C-Se-C Forms (SeMet, MeSeCys, γGluMeSeCys)**	**Se^0 (red)**
Mixed bacteria	3.78E-04				100%
Mixed diatoms	2.15E-03	15.1%	84.9%		
Ephydra larvae	6.38E-03			100%	
Ephydra pupa	5.22E-04			100%	
Ephydra adult 1	9.00E-04			100%	
Ephydra adult 2	1.08E-03		2.4%	97.6%	
Artemia eyes	2.42E-04			100%	
Artemia muscle	8.71E-05			100%	
Artemia cysts	9.79E-05		4.5%	95.5%	

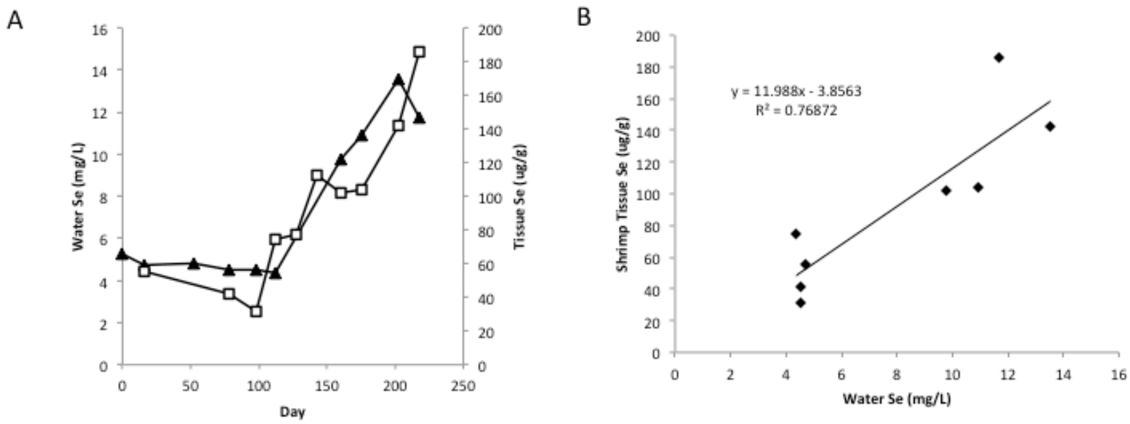

Figure 25. Total Se concentration in *A. franciscana* dry weight versus total Se concentration in water at the time of *A. franciscana* harvest (A). Correlation of *A. franciscana* Se tissue concentration to Se water content (B).

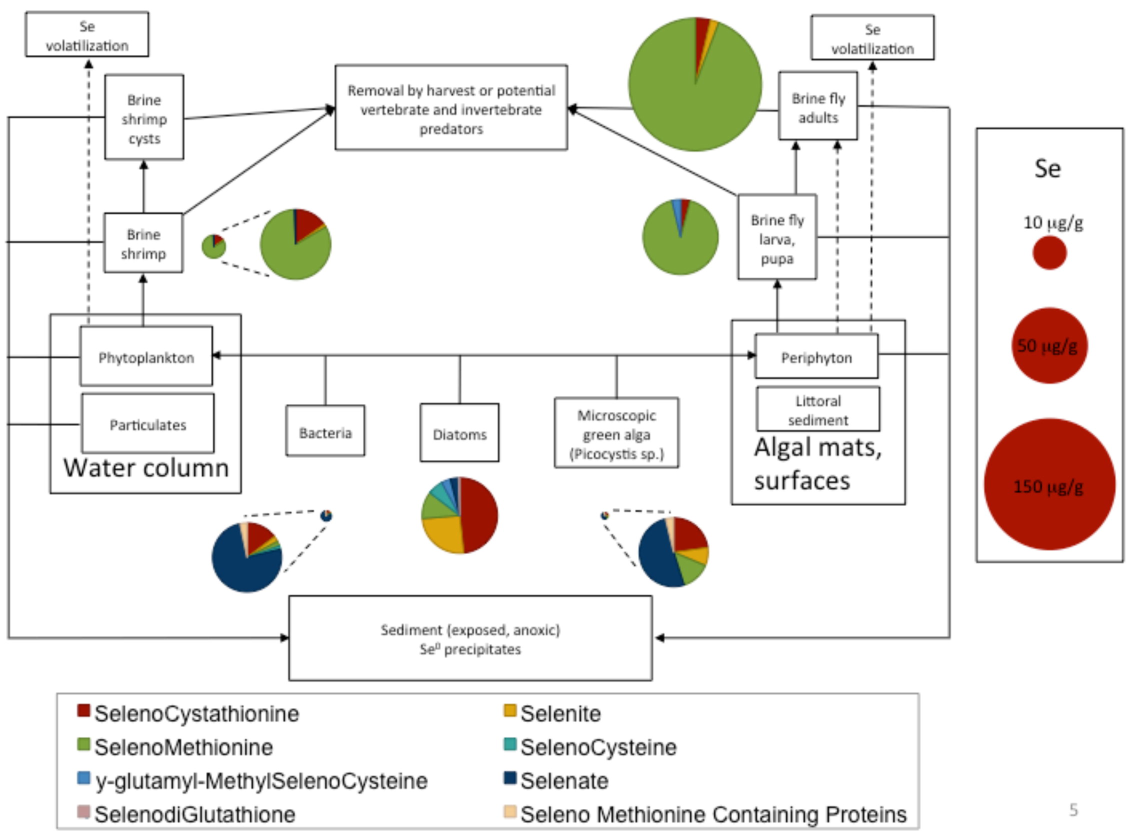

Figure 26. Community Se distribution - conceptual Se trophic levels model. Se species based on SAX ICPMS analysis. Size of each pie chart represents total Se concentration in respective samples. Note that bacteria and *Picocystis* samples were harvested from media with an ambient

concentration of 40 mg/L Se. All other samples were harvested from water with ambient concentration of approximately 9 mg/L Se.

Taken together, these results provided a complete model of the Se biochemical transformations that occur at each trophic level (Fig. 26).

Conclusions

The pilot-scale RRR selenium bioremediation test facility has demonstrated several important concepts to date. While laboratory experiments had shown that increasing salt concentration would limit *Cladophora* growth and survival (Table 1), we were not able to test if these effects held true at the field-scale size facility due to continuing drought conditions and lack of irrigation near the site. These prevented the generation of hypersaline water necessary to increase the total salinity in the pond at full capacity. Even at minimal water levels (i.e. below 1/3 capacity) water salinity only reached approximately 60 ppth, too low for efficient *A. franciscana* production or *Cladophora* growth mitigation (see Appendix Figures A1 and A3). At the same time, these conditions allowed the observation of *A. franciscana* population response to rapidly fluctuating conditions, and showed that *A. franciscana* numbers at temperatures above 20^{o}C respond most strongly to salinity and to a lesser degree to changes in pH.

The results of the mesocosm experiment indicated that the addition of carbon dioxide leads to an increase in algal biomass and resultant increase in brine shrimp numbers while the additions of organic carbon compounds did not have similar positive effect. High inorganic carbon concentration significantly increased carbon availability, lowered the pH, and therefore also increased availability of phosphorus and iron. The increase of those three nutrients resulted in an increase of algal biomass production. Since the algae biomass as shrimp food increased, the number of shrimp also increased. Several forms of nitrogen and organic carbon did not show significant correlation with algal biomass and shrimp production. However they did change water chemistry. Nitrite level was higher whereas nitrate level was lower in organic carbon-addition treatments. The microscopy and DGGE results showed that the algal communities in mesocosms were more similar to the communities in the pond than in microcosms. Therefore results from mesocosms were more practical and more similar to the pilot-scale pond than laboratory microcosms. We used DGGE as a molecular method to analyze microalgal and bacterial communities.

An experiment to optimize algae concentration and to study effect of inoculation with isolated strain on total biomass in mesocosms was performed in UC Davis greenhouse under controlled temperature. Using native isolated algal strain, *Picocystis salinarum* RRR1, to increase algae biomass production in RRR water is more promising than introducing new strains that may not survive in the drainage water with high Se present. Laboratory experiments are providing essential data for optimizing culture conditions to enhance algal production. The mesocosm experiment with addition of *Picocystis sp.* biomass showed establishment of a promising system between algae and brine shrimp, which could be used for producing brine shrimp as biodiesel feedstock.

A critical point of Se ecotoxicity studies is that chronic toxicity resulting from dietary Se uptake and foodchain transfer represents a far greater problem than acute toxicity associated with direct water exposure. Waterborne Se concentration is not a good indicator of Se risk due to extensive biotransformations and foodchain transfers. In all studies to date, including this one, it has been shown that macroinvertebrates that form the food for fish, birds and other animals, bio-accumulate Se exclusively in organic, highly bioavailable forms. Our findings showed that inorganic Se, occurring in the drainage water as selenate, was first accumulated by the microorganisms in our pond system - microalgae, bacteria and diatoms, reduced into selenite, selenide, or elemental Se, and some was metabolized into organic Se selenomethionine. Organisms in the upper trophic levels accumulated and bioconcentrated Se from this microscopic food sources and further metabolized the different Se-forms predominately into selenomethionine. The highest Se concentrations were observed in the adult brine flies, swarming on the water surface and pond edges, followed by the aquatic brine-fly larva and brine-shrimp. New information describing the range and distribution of Se species within organisms living in contaminated environments will lead to improved ecosystem management practices. In small quantities Se is a dietary requirement for mammals. The concentration of bioavailable Se by invertebrates such as *A. franciscana* could represent a relatively inexpensive source of Se as a supplement for Se deficient humans and animals, at the same time transforming Se-contaminated agricultural wastewater into a useful resource.

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Appendix A – RRR racetrack pond physical parameters

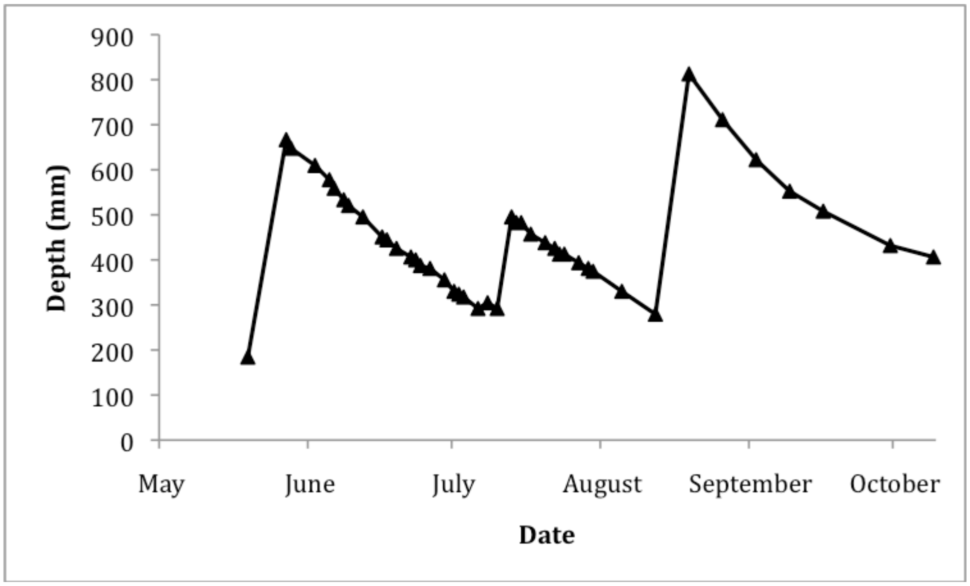

Figure A1. Pond water depth over the 2010 monitoring period. Water lost due to evaporation or pond cleaning was replaced with low-salt, low-Se aqueduct water due to drought conditions.

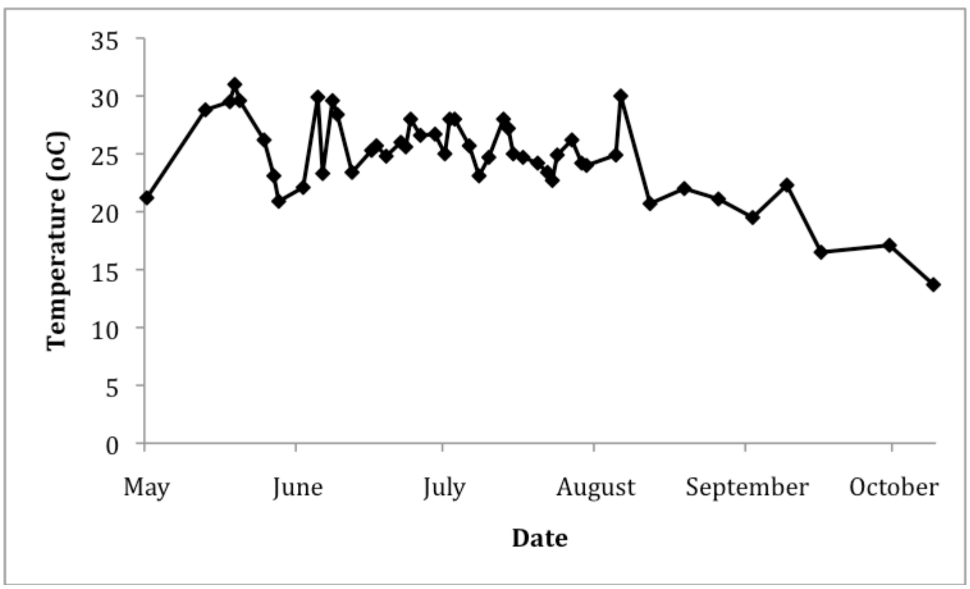

Figure A2. Daily water temperature profiles for RRR racetrack pond for the 2010 monitoring period.

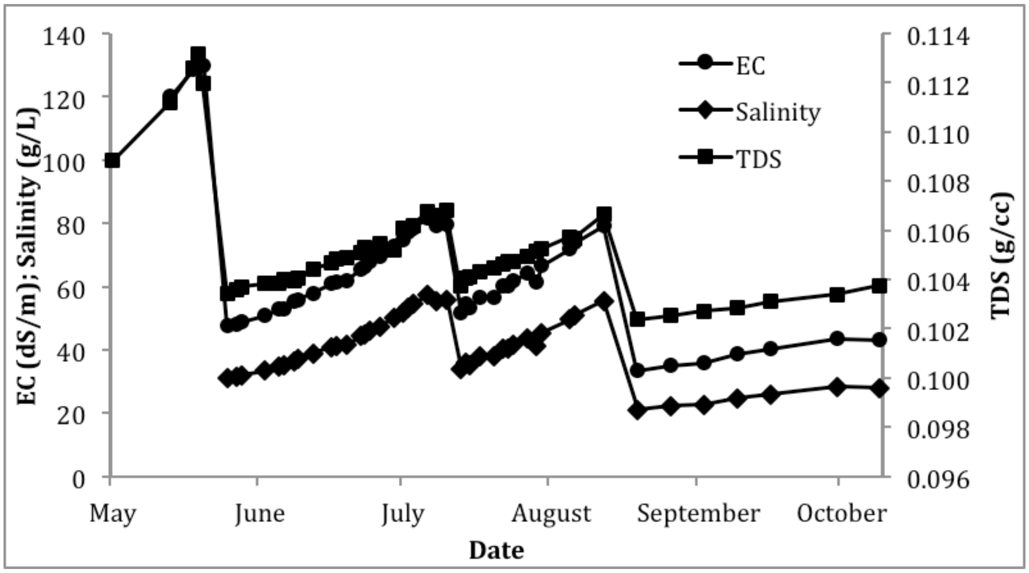

Figure A3. Salinity, EC and TDS of RRR water over the 2010 monitoring period. Due to a combination of drought conditions and limited irrigation in the adjacent fields, we were not able to supplement the pond with highly saline IFDM water. Water salinity ranged from 20-60 g/L.

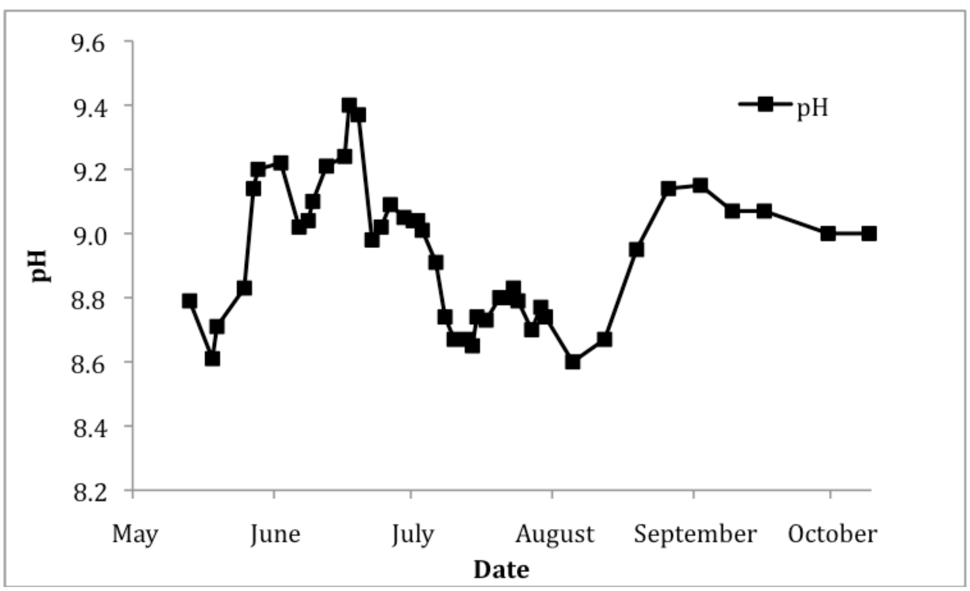

Figure A4. pH of RRR water over the 2010 monitoring period.

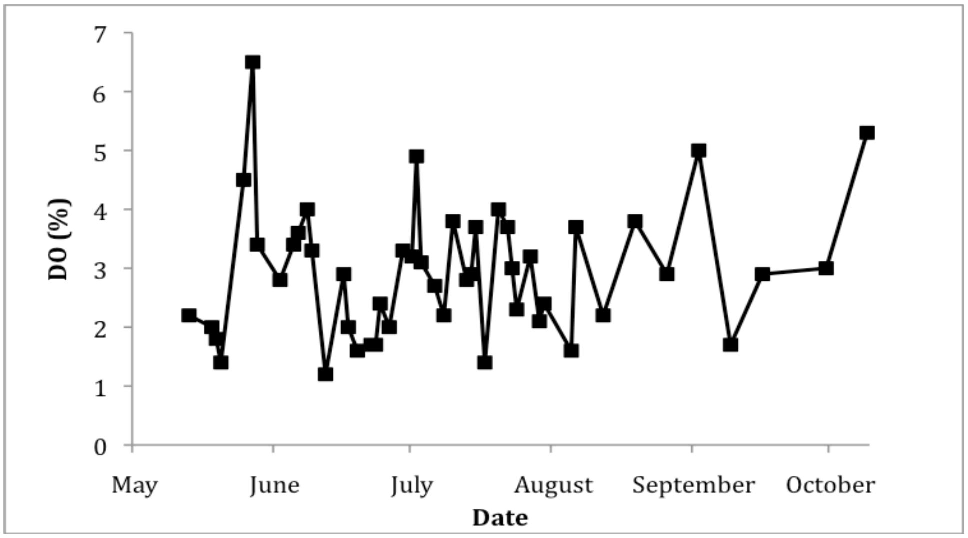

Figure A5. Dissolved oxygen (DO) of RRR water over the 2010 monitoring period. Due to mostly lower water level than previous years, paddlewheel alone was able to maintain sufficient DO.

Appendix B - Se speciation Materials and Methods

2.1 System Design, Location and Water Chemistry Parameters

Integrated on-Farm Drainage Management (IFDM) system cycles drainage water through a series of salt tolerant crops with the objective of reusing the saline water to produce marketable crops. At RRR the drainage water has been used in several irrigation cycles, irrigating progressively more salt tolerant crops, the reduced volume of drainage water is collected in the final sump system, solar evaporated and used in the racetrack pond. The agricultural drainage water was very high in dissolved salts and metals: Se 6-12 mg L^{-1}, SO_4^{2-} approximately 65 g L^{-1}, Na^+ 17 g L^{-1}, Mg^{2+} 1 g L^{-1}, Ca^{2+} 0.5 g L^{-1}, K^+ 51 mg L^{-1}, and B 142 mg L^{-1}. The water also typically contained approximately 300 mg L^{-1} NO_3^{2-}, but was low in NH_4^+ 1-3 mg L^{-1}, Fe 0.5 mg L^{-1}, P 0.8 mg L^{-1}, Si 0.9 mg L^{-1} and V 0.01 mg L^{-1}. Other dissolved metals were below their respective detection limits: Zn, Mn, Cd, Cr, Ni and Al <0.05 mg L^{-1}; Cu and Pb <0.1 mg L^{-1}. Sequential application of the drainage water reuses 90% of the wastewater produced at RRR and there is no disposal into rivers or evaporation basins. Red Rock Ranch, located near Five Points, California, contains an experimental site where a water wheel-driven racetrack pond was built to remediate Se-laden waste water and produce Se-enriched brine shrimp.

2.2 Sample growth and preparation

2.2.1 Microorganisms

Bacterial growth

Bacterial culture medium consisted of 0.2 mm filter (Millipore) sterilized RRR drainage water diluted to 80 mg/L salinity, 1% v/v glycerol (Fischer), 2% w/v tryptone (Difco), 20 mgL^{-1} Fe-gluconate (Sigma-Aldrich), 6 mg L^{-1} NaH_2PO_4 (Sigma-Aldrich). For culturing, 10 ml of RRR drainage water filtered through 5 mm sterile syringe filter (Millipore) was added to 90 ml of media. Cultures were incubated overnight at 25^{o}C in the dark on an Orbit shaker-incubator (Lab-Line) at 100 rpm.

Microalgae growth

Picocystis sp. growth medium consisted of RRR agricultural drainage water at approximately 80 g/L salinity equivalent amended with Na_2SeO_4 to 45 mgL^{-1}. 500 ml culture flasks containing 100 ml culture media were incubated for one week under 30W compact fluorescent lights on a MaxQ2000 orbital shaker (Thermo Scientific) at 90 rpm.

Diatom growth

A mixed diatom culture was isolated by a modification of the protocols of Bruckner and Kroth (Bruckner et al. 2009). Diatom medium consisted of RRR agricultural drainage water at approximately 80 g/L salinity equivalent amended with Na_2SeO_4 to 40 mg/L. Chloramphenicol 27 μg/ml, streptomycin at 135 μg/ml and ampicillin at 270 μg/ml were used as selective agents. Diatom inoculum was prepared by filtering 100 ml RRR water through 2 μm filter paper. Filter paper was washed with 100 ml 80 ppt sulfate buffer. Washed filter was placed in 10 ml Diatom media and gently vortexed for 30 s. 5 ml inoculum was used per 100 ml solution. Flasks were incubated for one month under 30W compact-fluorescent lights on a MaxQ2000 orbital shaker (Thermo Scientific) at 90 rpm.

Microbial sample harvest

Samples (bacteria, microalgae and diatoms) were harvested by centrifugation at 5856 g for 20 min at 23^{o}C. Cells were washed three times in 5 ml 80 ppt sulfate buffer. Pellets in 2 ml microcentrifuge tubes were flash frozen in liquid nitrogen and stored at -80^{o}C until analysis.

2.2.2 Macroinvertebrates

Sample harvest and preparation from RRR racetrack pond

E. cinera larvae and pupa were collected in 1 L plastic water sampling bottles. Adult flies were collected by trapping in empty 50 ml Falcon tubes. *A. franciscana* were collected in 1 L plastic water sampling bottles. All macroinvertebrate samples (except for adult fly) were washed by transfer to buchner filter funnels lined with 90mm Whatman No 1. circular filter papers and vacuum filtered using a sterile 80 mg/ml sodium sulfate wash solution, which was vacuumed through completely before samples were flash frozen in liquid nitrogen. *E. cinera* adults were directly flash frozen in liquid nitrogen. All samples were then stored at -80^{o}C until analysis.

2.3 Selenium analysis

2.3.1 Quantification of total Se

Aquatic organisms were removed from the freezer and were digested with HNO_3, H_2O_2, and HCl (Bañuelos and Akohoue 1994). Mineral inorganic elements, including Se, were analyzed by an inductively-coupled plasma mass spectrometer (Agilent 7500cx, Santa Clara, USA). The National Institute of Standards and Technology (NIST) Wheat Flour (SRM 1567; Se content of 1.1±0.2 μg g^{-1} DM) and two internal soil standards (sediment collected from Kesterson Reservoir, CA, with a total Se content of 7.5 ±0.25 and 25 ±0.87 mg kg^{-1}) were used as the Se quality control standards. The Se recovery rates of the Se in standard materials after acid digestion were over 94%.

2.3.2 Chemical speciation of soluble Se

Sample extraction for SAX-HPLC-ICPMS

Aqueous Se compounds were separated from insoluble Se compounds based on the extraction protocol described in Bañuelos et al. 2011. Duplicate samples (0.1-0.2 g) were placed in separate 40 mL glass vials equipped with Teflon caps. Protease XIV (50 mg) and water (10 mL) were added to one vial. The other vial received 17 mL of methanol (ultrapure) and no protease nor water. The paired samples were vortexed, and the protease sample set was incubated in a shaker for 20 h at 37 °C, while the sample set with methanol was maintained overnight at 4 °C.

Methanol (17 mL) was then added to the protease-digested samples and water (10 mL) was added to the non-protease digested samples. All samples were mixed thoroughly and refrigerated overnight at 4 °C. Following this, chloroform (8.5 mL) was added to all vials, which were shaken vigorously, and then refrigerated again at 4 °C overnight. Vials were tapped to break emulsions between the upper aqueous (methanol-water) phase and the lower organic phase. The aqueous phase (~27 mL) was then carefully transferred with a Pasteur pipette to a 50 mL conical centrifuge tube. One quarter of the aqueous phase (~6.8 mL) was then pipetted into 50 mL ICP digestion tubes, evaporated with a heating block at 50 °C (~300 min), acid digested, and analyzed for total Se by ICPMS. The entire non-aqueous chloroform phase remaining in the original 40 mL glass vial was also evaporated with a heating block at 50 °C (~300 min), acid digested, and analyzed for total Se by ICPMS. Percentage of Se in the aqueous phase after extraction was calculated as (total Se in aquaeous phase)/[(total Se in aquaeous phase)+(total Se in chloroform)x100]. The remaining aqueous phase (~ 20.3 mL) was dried *in vacuo* with a refrigerated centrifugal speed vacuum, re-suspended in 2.5 mL water, and stored at -80 °C.

Waters Sep-Pak® Classic C18 cartridges (360mg 55-105μm) were used for final clean-up of the aqueous concentrates. Each cartridge was cleaned by flushing 10 mL of methanol and 5 mL ultrapure water in succession. Concentrates (2.5 mL) were thawed, vortexed, 11 μL of 88% formic acid was added, and transferred with Pasteur pipette to the cartridge. Elution with methanol (3 mL) was into a 50 mL conical tube. Eluant was then dried *in vacuo* as above, dissolved in water (1.5 mL), and transferred into Agilent screw-top glass HPLC vials and frozen until SAX-HPLC-ICPMS analysis.

SAX-HPLC-ICPMS

An Agilent 1200 HPLC separations module equipped with a Hamilton PRPX-100 strong anion exchange analytical column (10mm particle size- 25cm length x 4.1mm internal diameter) and an Agilent 7500 ICPMS was used for Se speciation of aqueous extracts as described by Bueno et al. (2007). A single analysis (30 mL injection) was conducted for each of the aqueous extracts. Chromatographic separation of all Se compounds was achieved with an isocratic mobile phase of 5 mM ammonium citrate buffer (pH 5.2) with 2% methanol at a flow rate of 1mL/min and ^{78}Se was monitored in real time using the ICPMS. Retention times of Se containing peaks were compared to the authentic standards (listed below).

Selenium standards

Sodium selenate (Na_2SeO_4), sodium selenite (Na_2SeO_3), selenocystine (CysSe-SeCys), and seleno-methionine (SeMet) were obtained from Sigma-Aldrich. Methyl-seleno-cysteine (MeSeCys), gamma-glutamyl-methyl-selenocysteine (gGMeSeCys), seleno-cystathionine (SeCyst) and seleno-diglutathione ($SeGSH_2$) standards were obtained from PharmaSe. Seleno-cysteine ($SeCys^-$) was obtained by reducing CysSe-SeCys at 50 °C for 48 hr in 50 mM Dithiothreitol at a 1:1 molar ratio.

2.3.2 Se distribution and chemical speciation

μXRF/μXANES

The distribution of Se, Ca and Zn were determined using micro focused X-ray fluorescence (μXRF) mapping and the local speciation was determined using micro Se K-edge X-ray absorption near-edge structure (μXANES) spectroscopy. Samples were washed to remove any external Se, flash frozen in liquid nitrogen, and taken to beamline 10.3.2 of the Advanced Light Source at the Lawrence Berkeley National Laboratory for analyses (Marcus et al. 2004). Frozen samples were transferred onto a Peltier stage kept at –27°C to minimize radiation damage. Micro-XRF elemental maps were recorded at 13 keV, using a 15-μm (horizontal) x 6-μm (vertical) beam, a 15-μm x 15-μm pixel size, and 50-ms dwell time per pixel, except for the brine fly pupa, which was imaged using a 25-µm x 25-µm pixel size. The chemical forms of Se in specific areas of increased accumulation were further investigated using Se K-edge µXANES. Maps and spectra were recorded with a seven-element Ge solid-state detector (Canberra). Spectra were deadtime corrected, pre-edge background subtracted, and post-edge normalized using standard procedures (Kelly et al. 2008). Red Se (white line maximum set at 12660 eV) was used to calibrate the spectra. Least squares linear combination fitting (LCF) of the Se XANES spectra was performed in the 12630- to 12850-eV range using a library of nine standard selenocompounds as described in Freeman et al., 2006. The error on the percentages of species present is estimated to be ±10%. Aqueous solutions of the various selenocompounds listed above were used as standard materials, while red and gray elemental Se were a gift from Dan Strawn (University of Idaho). All data processing and analyses were performed with a suite of custom LabVIEW programs available at the beamline.

Bulk XANES

Samples were removed from storage at -80 °C, macerated in liquid nitrogen using a mortar and pestle, packed into 2-mm path length sample cells, and maintained in liquid nitrogen for bulk Se K-edge XANES analysis. Spectra were collected on beamline No. 9-3 at the Stanford Synchrotron Radiation Lightsource (SSRL) with the storage ring operating at 3 GeV, using an upstream Rh-coated collimating mirror, a Si(220) double-crystal monochromator, and a downstream Rh-coated focusing mirror. The incident X-ray intensity was monitored using a N_2-filled ionization chamber. XANES spectra were recorded by monitoring the Se K_α fluorescence using a 30 element germanium detector equipped with Soller slits and an arsenic filter. During data collection, samples were maintained approximately at 10 K in a liquid helium flow cryostat, as described in Andrahennadi et al. (2007). Fluorescence spectra were also collected on dilute aqueous solutions of standard Se species buffered at pH 7, including the inorganic Se forms as SeO_4^{2-} and SeO_3^{2-}, the organic forms of SeMet, trimethylselenonium ion, CysSe-SeCys, $SeCys^-$, and $SeGSH_2$. S and solid red elemental Se was also measured in transmission mode. Experimental spectra were calibrated with respect to the spectrum of hexagonal Se, which was placed downstream of the sample and measured simultaneously in transmission (first inflection point set at 12658 eV). Background subtraction, normalization calibration and further data analyses were carried out according to standard procedures using the EXAFSPAK program suite available at the beamline. Experimental spectra were analyzed by least-squares linear combination fitting (Pickering et al., 1999) to extract the percentage contribution from each species (assuming that all species present were represented in the set of standards). The percentage contribution of a standard spectrum to the sum is equal to the percentage of total Se present in that chemical form.

Sample preparation for Total Se Analysis by spectrofluorimetry

To analyze for total waterborne Se, 10 mL of sample water was placed in a Falcon tube and centrifuged at 5000 rpm for 10 minutes. Immediately after centrifugation, 2mL of the supernatant was transferred to a glass scintillation vial. The pH was adjusted to <2 with concentrated HCl and the samples were stored in the dark at room temperature until analysis.
Brine shrimp tissue Se analysis included washing the collected biomass with deionized water. Excess water was blotted from the biomass. The shrimp were then folded in tin foil, frozen in liquid nitrogen and stored in a brown paper bag at –80°C until lyophilization and Se analysis could be performed. Between 1.5-2.5 mg of the dry shrimp biomass was digested overnight in 1.6 mL concentrated HCl. An aliquot (20-200 µL) of the digestate was added to a GC vial for Se analysis, as described below.

Total Se Analysis by Spectrofluorimetry

Total Se in water, tissue and volatile matrices was analyzed using a micronitric acid digestion coupled with fluorescence detection (Fan*,* 1998b). An aliquot (10-200 µL, depending on the matrix and estimated concentration) of sample media was pipetted into a 2 mL Target GC vial (Agilent). The samples were digested in 500µl of concentrated HNO_3, 10 µL concentrated H_2SO_4, and 50 µL 5% $K_2S_2O_8$ at 100 °C for 30 minutes. The temperature was raised to 130 °C and the samples remained at this temperature until the volume was less than 40 µL. The samples were then reduced using 100 µL 6N HCl at 110 °C until the volume was less than 40 µl. One milliliter of 0.1N HCL was then added to dilute the samples. Next, 100 µL the derivatizing agent, 0.125% diaminonaphthalene-hydrochloride (Aldrich), was added along with 50 µL of 20mM EDTA + 10% hydroxylamine sulfate and 500 µL cyclohexane. The derivatization mixture was sealed with a Teflon-lined septum cap and incubated at 45 °C in the dark for 30 min. The samples were shaken vigorously, allowed to settle, and 250 µL of the hexane layer was transferred to a glass microplate. The fluorescence intensity was measured using a spectrofluorimeter equipped with a Genios microplate reader (Phenix Research Products). Se analysis included 2 replicates and 1 spiked sample with standards errors < 15%. A standard curve consisted of 9 SeO_2 (Fisher Scientific) solution standards ranging from 0 to 400 mg L^{-1} for waterborne and 0 to 60 mg L^{-1} for tissue and volatile-Se analyses.

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